University of Alberta

Homocysteine Metabolism as a Response to Aging and Folic Acid Intake in Fisher 344 Rats

by

Anastasia Katherine Nimchuk

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Abstract

Susceptibility to cardiovascular disease (CVD) increases throughout the aging process, as do the levels of its risk factors, such as plasma homocysteine (hcy) and cholesterol. Homocysteine metabolism depends on vitamin B_6 dependent trans-sulfuration of hcy to cysteine (cys), and folate and vitamin B_{12} dependent remethylation of hcy to methionine. Using Fisher 344 (F344) rats, the present study was undertaken to determine the effect of age on hcy metabolism. The relationships between age, plasma concentrations of hcy, cholesterol and cys, as well as the blood levels of the vitamins B_6, B_{12}, and folate were assessed in F344 rats aged 12, 18 and 24 months. The 24 month-old rats had hcy concentrations 26-37% higher than the 12 and 18 month-old ($p<0.05$) and both creatinine and total cholesterol concentrations were directly associated with age and hcy levels ($p<0.05$). Folate levels were lower ($p<0.05$) in the 18 and 24 month-old than in the 12 month-old rats. To test whether improving folate status might attenuate the age-associated increase in hcy and cholesterol levels, a second experiment was conducted involving F344 rats aged 12 and 24 months. The 24 month-old rats received diet, containing either physiological (3.57 mg/kg diet) or supplemental (35.7 mg/kg diet) dose levels of folic acid for 4 weeks. The supplemental intake of folic acid improved ($p<0.05$) the age-associated decrease in plasma folate levels and prevented ($p>0.05$) the hyperhomocysteinemic effect of aging. The age-associated increase in cholesterol and creatinine levels, however, remained unchanged ($p>0.05$) in the presence of folic acid supplementation. Overall, the results of this study confirm that age is an important risk factor for hyperhomocysteinemia. This may be a reflection of a combination effect of reduced renal function and remethylation of hcy to cys, as indicated by increased creatinine and decreased folate levels in the plasma of older rats. The results also suggest that the age-induced hyperhomocysteinemia can be prevented by folic acid supplementation.

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled ***Homocysteine Metabolism as a Response to Aging and Folic Acid Intake in Fisher 344 Rats*** in partial fulfillment of the requirements for the degree of Master of Science in Nutrition and Metabolism.

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List of Abbreviations

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
ARIC	Atherosclerosis Risk in Communities
ATP	Adenosine triphosphate
BUN	Serum urea nitrogen
BUPA	British United Provident Association
BW	Body weight
CBS	Cystathionine β-synthase
CHD	Coronary heart disease
CVD	Cardiovascular disease
Cys	Cysteine
DAP	Dihydroxyacteone phosphate
DNA	Deoxyribonucleic acid
F344	Fisher 344
FAD	Flavine adenine dinucleotide
G-1-P	Glycerol-1-Phosphate
G1P-DH	Glycerol-1-Phosphate Dehydrogenase
GK	Glycerol Kinase
Hcy	Homocysteine
HMG-CoA	3-Hydroxy-3-Methylglutaryl Coenzyme A
HPLC	High Performance Liquid Chromatography
INT	2[-P-Iodophenyl]-3-P-Nitrophenyl-5-Phenyltetrazolium Chloride
INTH	Formazan
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
Met	Methionine
MI	Myocardial infarction
MMA	Methylmalonic acid
MRFIT	Multiple Risk Factor Intervention Trial
MTHF	5,10,-methylene tetrahydrofolate
MTHFR	5,10,-methylene tetrahydrofolate reductase
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NHANES	National Health and Nutrition Examination Survey
NIA	National Institute of Aging

NIH	National Institute of Health
NO	Nitric oxide
OPA	o-phthaldialdehyde
PA	Pyridoxic acid
PCV	Packed cell volume
PLP	Pyridoxal-5'-phosphate
RBC	Red blood cell
RNA	Ribonucleic acid
RNI	Recommended Nutrient Intake
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SAS	Statistical Analysis System
SBDF	7-fluorobenzo-2-oxa1,3-dizole-4-sulfonate
TBIL	Total bilirubin
VITAL	Vitamin, Teachers and Longevity
WBC	White blood cells

CHAPTER 1: INTRODUCTION

Metabolism of homocysteine

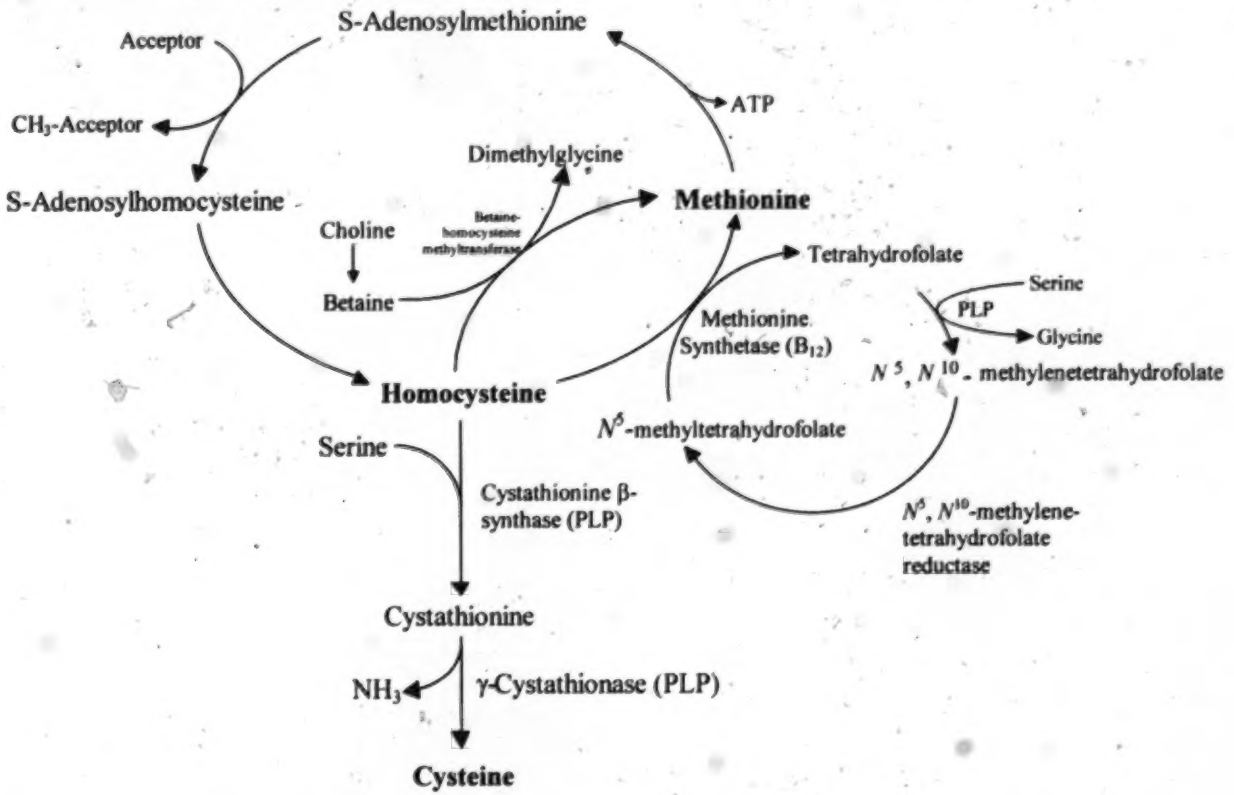
Homocysteine is a sulfur-containing amino acid derived from methionine, an essential amino acid (Figure 1). Methionine is continuously converted, in an ATP dependent reaction, to s-adenosylmethionine (SAM), which functions as a methyl donor for a variety of methylation reactions; including the methylation of DNA, RNA and protein (Stipanuk, 1986). Following methyl group donation, SAM is converted to S-adenosylhomocysteine (SAH). However, all SAM dependent transmethylation reactions are inhibited by SAH, thus, the subsequent metabolism of SAH is vital (Finkelstein, 1998). Adenosylhomocysteinase catalyzes the hydrolysis of SAH to homocysteine and adenosine. Homocysteine is metabolized by one of two major pathways: The first is the remethylation of homocysteine to methionine and the second is the catabolic trans-sulfuration of homocysteine to cysteine via cystathionine.

The generation of methionine from homocysteine functions to conserve the essential amino acid. The enzyme responsible for this reaction, methionine synthetase, requires vitamin B_{12} as a cofactor and methyltetrahydrofolate as a substrate. Methylenetetrahydrofolate reductase, a vitamin B_2 (FAD) dependent enzyme, is responsible for the reduction of methylenetetrahydrofolate (MTHFR) to methyltetrahydrofolate. Another methionine-conserving means of disposing of homocysteine is via betaine-homocysteine methyltransferase. This hepatic and renal enzyme catalyzes the remethylation of homocysteine to methionine using betaine (trimethylglycine), which can be derived from the oxidation of dietary choline (Stipanuk, 1986). As well, it can be synthesized through successive methylations of phosphatidylethanolamine (Stipanuk, 1986). Hepatic folate and betaine remethylation reactions appear to be of equal importance in rats, but in humans the folate dependent remethylation dominates (Finkelstein and Martin, 1984; McKeever et al., 1991). As dietary intake of methionine alone is not sufficient to sustain the amount of SAM required for methylation reactions, the *de novo* synthesis of methionine via the remethylation of homocysteine is essential (Parnetti et al., 1997).

When methionine is in excess, conservation is not necessary; thus, homocysteine is directed towards the catabolic trans-sulfuration pathway. This pathway irreversibly converts homocysteine to cysteine in two steps. First, the pyridoxal-5'-phosphate (PLP)-dependent cystathionine β-synthase (CBS) catalyzes the replacement of the β-OH group of serine with homocysteine to form cystathionine. Another PLP-dependent enzyme, cystathionase

(cystathionine γ-lyase), subsequently catalyzes the cleavage of cystathionine to cysteine and α-ketobutyrate (Stipanuk, 1986). The PLP dependent enzyme CBS is an important regulatory point in the metabolism of homocysteine. In addition to catalyzing an irreversible reaction, it undergoes up-regulation in the presence of excess methionine (Finkelstein and Martin, 1984; Masser et al., 1994). S-adenosylmethionine allosterically activates CBS by reducing the Km for homocysteine approximately eight fold, without affecting the V_{max} (Finkelstein et al., 1975). Additional regulation by SAM occurs at betaine-homocysteine methyltransferase and at MTHFR; when methionine is in excess the increased SAM produced inhibits remethylation by inactivating these key enzymes (Masser et al., 1994).

Figure 1: Methionine and homocysteine metabolism

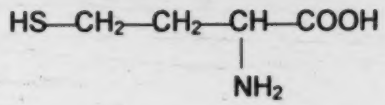


Adapted from: (Selhub and Miller, 1992)

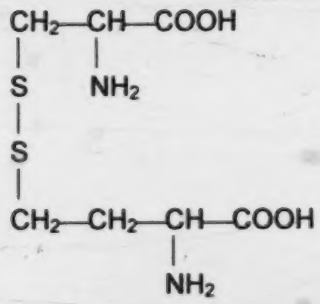
Plasma homocysteine and its determinants

The sulfhydryl group of homocysteine renders it highly susceptible to oxidation at physiologic pH. Only trace amounts (1%) of plasma homocysteine exist in its reduced form *in vivo*. Approximately 70-90% of the homocysteine in plasma is bound to plasma proteins; the remainder is rapidly oxidized to the disulfides cysteine-homocysteine and homocystine (Jacobsen, 1998; Ueland et al., 1996). Thus, the term plasma/serum homocysteine is used in reference to all 3 forms of the amino acid (Figure 2).

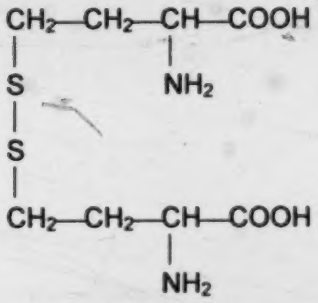
Figure 2: Molecular species of homocysteine found *in vivo*



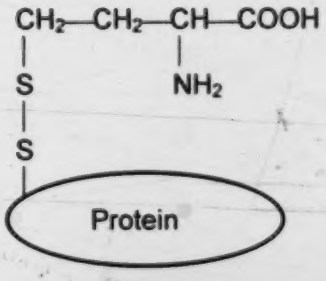
Homocysteine (reduced)



Cysteine-homocysteine disulfide (oxidized)



Homocysteine-homocysteine disulfide (Homocystine, oxidized)



Protein-bound homocysteine (oxidized)

Genetic determinants of plasma homocysteine

Homocystinuria is most often used to describe a variety of inborn errors of metabolism that lead to severe hyperhomocysteinemia. The most common cause of homocystinuria is homozygous CBS deficiency, which occurs approximately 1/200 000-300 000 births (Skovby, 1989). Plasma homocysteine levels in these patients are often 5 to 50 times greater than the normal range and are usually accompanied by elevated plasma methionine levels (Cattaneo et al., 1999). Other forms of homocystinuria include severe defects of MTHFR, MS, and inborn errors of cobalamin metabolism. These conditions are often associated with the premature development of vascular and thromboembolitic diseases.

Genetic defects can also lead to the development of less severe hyperhomocysteinemia. Heterozygous CBS deficiency occurs in 0.5 to 1.5% of the general population (Boers et al., 1985; Cattaneo et al., 1999). Fasting homocysteine levels are often normal in these patients, however the homocysteine levels are elevated following a methionine load (Boers et al., 1985). Genetic studies of patients with vascular disease indicate that heterozygous CBS deficiency occurs infrequently in these patients (Boers et al., 1985; Kluijtmans et al., 1996; Kozich et al., 1995). Moreover, the prospective Atherosclerosis Risk in Communities (ARIC) Study found no association between heterozygous CBS mutations and coronary heart disease (Folsom et al., 1998).

The most common genetic defect in homocysteine metabolism is the thermolabile variant of MTHFR. Kang et al (1988) were the first to describe this variant, which is the result of a specific cytosine to thymidine (C→T) substitution at nucleotide position 667. The result of the point mutation is the substitution of valine for alanine in the methylenetetrahydrofolate binding site (Frosst et al., 1995). The thermolabile variant is so named because it is characterized by increased sensitivity of the protein to temperature (Kang and Wong, 1996b). There is a population-based difference in the reported frequency of homozygosity for the C667T mutation (Table 1).

The hyperhomocysteinemic effect of the thermolabile MTHFR genotype appears to be related to folate sufficiency (Bailey and Gregory, 1999; Malinow et al., 1998). According to the US Physicians Health Survey, homozygotes with the lowest plasma folate levels are associated with the highest plasma homocysteine levels (Stampfer et al., 1992). Jaques et al. (1996) reported that homozygotes and individuals with a normal genotype who had high plasma folate levels had

similar homocysteine levels. However, among subjects with low plasma folate levels homocysteine concentrations were 24% higher in homozygotes than in those with normal MTHFR (Jacques et al., 1996). These findings have lead to the belief that the intake of folate required to normalize plasma homocysteine levels is greater in homozygotes with the C667T mutation (Rosenberg and Rosenberg, 1998). Studies examining the relationship between the C667T genotype and cardiovascular disease appear to be contradictory, possibly due to the complicating effect of the gene-nutrient interaction on homocysteine levels (Brattstrom et al., 1998; Kang, 1996a; Ma et al., 1996; Verhoef et al., 1997a; Wilcken et al., 1996).

Table 1: Reported prevalence of C667T mutation in various control populations

Population	Prevalence of C667T mutation (%)	Reference
African Americans	1.4	(Austin et al., 1997; McAndrew et al., 1996)
Dutch	5	(van der Put et al., 1995)
Japanese	10	(Morita et al., 1997)
Australian	11	(van Bockxmeer et al., 1997)
French Canadian	13	(Blom, 1998)
Italian	15-20	(de Franchis et al., 1996)

Physiologic determinants of plasma homocysteine

Premenopausal women generally have lower homocysteine levels than men of the same age (Jacques et al., 1999; Nygard et al., 1995). This appears to be largely due to hormonal influences, as plasma homocysteine levels increase after menopause and decrease with oral estrogen administration in men (Andersson et al., 1992b; Giri et al., 1998; van der Mooren et al., 1997). Differences in homocysteine concentrations between genders may also be partly attributed to lower plasma levels of vitamins B_6, B_{12} and folate in men (Lussier-Cacan et al., 1996; Selhub et al., 1993).

Age, in addition to gender, is also correlated with plasma homocysteine levels. Numerous epidemiological studies have observed increases in homocysteine levels with age (Jacques et al., 1999; Nygard et al., 1995; Selhub et al., 1993). According to the National Health and Nutrition Examination Survey (NHANES III), involving 3766 males and 4819 females aged 12 to >80 years, plasma homocysteine concentrations increase more rapidly with aging in adolescent and young adult males compared to females of the same age. Between the ages 30-49 years only small differences exist in homocysteine concentrations across age groups of either gender. However, after 50 years of age the magnitude of the age-related rise in homocysteine levels increases with age in both sexes but the rise is faster in females than males. The Hordaland Homocysteine Study (Nygard et al., 1995) as well as the Framingham Heart Study (Selhub et al., 1993) made similar observations. Furthermore, the prevalence of high homocysteine concentrations (>14 μmol/L) was found to be 29.3% for the entire cohort (67 to 96 years of age) and greater than 40% for individuals aged 80 years and older (Selhub et al., 1993). The reasons for age-related trends in plasma homocysteine are not fully understood. Possible mechanisms for this increase include declining renal function (Norlund et al., 1998), decreased activity of the enzymes regulating homocysteine concentrations (Ubbink, 1994) and impaired biochemical status of vitamins involved in homocysteine metabolism (Joosten et al., 1996; Joosten et al., 1993; Selhub et al., 1993).

Plasma homocysteine levels appear to be strongly influenced by renal function. There is a positive relationship between fasting homocysteine levels and serum creatinine; and most patients (85-100%) with end-stage renal failure are hyperhomocysteinemic (Bostom et al., 1997b; Robinson et al., 1996). The exact cause of hyperhomocysteinemia in renal failure is not known. The kidney does not appear to play a major role in homocysteine excretion since very little homocysteine is found in the urine (Bostom et al., 1995; Refsum et al., 1985). It has been shown, in rats, that the kidneys remove 20% of plasma homocysteine in a single pass, indicating intra-renal metabolism (Bostom et al., 1995). Extrapolation of these data suggests that renal uptake and metabolism could account for approximately 70% of daily homocysteine elimination in humans (Bostom et al., 1997b). However, van Guldener et al (1998) found no arteriovenous difference in plasma homocysteine, demonstrating a lack of renal homocysteine extraction in humans. The authors hypothesized that hyperhomocysteinemia occurs in end-stage renal failure because renal protein breakdown is enhanced and the excess methionine released may be converted into homocystine in the liver; or that extra-renal homocysteine metabolism is impaired by factors related to uremia such as accumulation of uremic toxins which could inhibit the

homocysteine-metabolizing enzymes or interfere with the metabolism of folic acid (van Guldener et al., 1998).

In addition to renal failure, several other clinical conditions are associated with hyperhomocysteinemia. These conditions include: hypothyroidism (Nedrebo et al., 1998), lymphoblastic leukemia (Refsum et al., 1991), severe psoriasis (Refsum et al., 1989), rheumatoid arthritis (Hernanz et al., 1999; Roubenoff et al., 1997) and cardiac and renal transplantation (Arnadottir et al., 1996; Gupta et al., 1998).

Nutritional and lifestyle determinants of plasma homocysteine

The most important lifestyle influence on homocysteine level is diet (Tucker et al., 1996). Higher intakes of fresh fruits and vegetables, breakfast cereals (Oshaug et al., 1998; Tucker et al., 1996), protein (Stolzenberg et al., 1999) and the habitual use of multivitamin supplements are all inversely associated with homocysteine levels (Brattstrom et al., 1994; Nygard et al., 1995;). Intake of these elements likely protects against hyperhomocysteinemia by influencing vitamin status, which is clearly influences homocysteine levels. If there is a deficiency in any one of the B-vitamins involved in homocysteine metabolism the risk of mild hyperhomocysteinemia increases. Epidemiological studies indicate that up to 66% of hyperhomocysteinemia in the population is associated with an inadequate blood level of one or more of these vitamins (Selhub et al., 1993; Selhub, 1999). The relationship between vitamin status and homocysteine levels is non-linear, with the strongest negative association at the lowest values in the distribution of vitamin status (Clarke et al., 1998; Lussier-Cacan et al., 1996; Pietrzik and Bronstrup, 1998; Selhub et al., 1993; Ueland et al., 1993). Plasma folate is the strongest vitamin determinant of plasma homocysteine in a healthy population (Pietrzik and Bronstrup, 1998; Selhub et al., 1993). Folate intake is also related to plasma homocysteine levels. At folate intakes of less than 350μg/day folate intake was found to be inversely proportional to plasma homocysteine levels (Pancharuniti et al., 1994; Selhub et al., 1993). There is also an association between vitamin B_{12} status in the general population and plasma homocysteine concentrations (Koehler et al., 1996; Naurath et al., 1995; Selhub, 1999). However, this association is not as statistically strong as the one between homocysteine and folate, likely because the prevalence of low vitamin B_{12} concentrations is not as great as the prevalence of low folate levels (Selhub, 1999). The relationship between vitamin B_6 status and homocysteine levels is less clear. Some studies have found a relationship between vitamin B_6 status and homocysteine levels (Selhub, 1997; Selhub et

al., 1993) while others have not (Dierkes et al., 1998; Miller et al., 1992; Pietrzik and Bronstrup, 1998; Ubbink et al., 1996; Verhoef et al., 1996). These divergent findings may be due to differences in methodology. Most studies measure fasting homocysteine levels, which may be normal even in cases of vitamin B_6 deficiency as homocysteine is recycled to methionine for use in protein synthesis and methylation reactions. However, when methionine is present in excess quantities, for example post-prandially or following a methionine load, trans-sulfuration of homocysteine to cysteine, via cystathionine-β-synthase and cystathionase, predominates. Thus, when vitamin status is suboptimal, transient hyperhomocysteinemia may occur, but, optimal vitamin B_6 status appears to be of particular importance after a methionine load (Brattstrom et al., 1994; Guttormsen et al., 1996; Miller et al., 1992; Nygard et al., 1995; Oshaug et al., 1998).

Many population studies have found that various lifestyle factors, other than diet, are also related to a shift of the distribution towards higher homocysteine levels. The Hordaland Homocysteine Study found that the consumption of 5 or more cups of caffeinated coffee per day was associated with elevated homocysteine levels (Nygard et al., 1998). Furthermore, according to the Vitamin, Teachers and Longevity (VITAL) study, a significant, positive, dose-response relationship was found between coffee consumption (up to 9 cups per day) and homocysteine levels (Stolzenberg et al., 1999). In contrast, Nieto et al (1997) found no relationship between coffee or caffeine consumption and homocysteine levels. A possible explanation may be that the latter study had a smaller sample size and fewer heavy coffee drinkers than either the Hordaland Homocysteine Study or the VITAL study. Cigarette smoking is another factor that has been reported to be associated with increased homocysteine levels (Nygard et al., 1998; Nygard et al., 1995; Oshaug et al., 1998), perhaps due to its effect on pyridoxal phosphate levels, as smokers have depressed pyridoxal phosphate synthesis (Nygard et al., 1995). Homocysteine levels are also elevated in cases of chronic, high alcohol consumption (Cravo et al., 1996) possibly due to poor vitamin status.

Pharmacological determinants of plasma homocysteine

A variety of drugs, especially those affecting the vitamins involved in homocysteine metabolism, are known to increase homocysteine levels. The antiepileptic drug phenytoin and the chemotherapeutic agent methotrexate increase homocysteine by interfering with folate availability (Kamen, 1997; Morgan et al., 1998). Vitamin B_{12} antagonists, such as nitrous oxide, and vitamin B_6 antagonists, such as theophylline, also elevate homocysteine levels (Brussaard et al., 1997a; Brussaard et al., 1997b; Koblin et al., 1990). L-dopa and the hypolipidemic agents

cholestyramine and niacin can also contribute to the development of hyperhomocysteinemia (Basu and Mann, 1997; Ueland and Refsum, 1989).

Homocysteine and cardiovascular disease

Cardiovascular disease is the predominant cause of morbidity and mortality in developed nations and accounts for almost 40% of deaths in Canada (Heart and Stroke Foundation of Canada, 1997). Approximately 25% of patients with cardiovascular disease do not have any of the established risk factors, thus the search for new risk factors is ongoing (Pasternak et al., 1996). The role of homocysteine in vascular disease was first suggested by McCully (1969) who observed autopsied evidence of severe atherosclerotic disease in children who died from homocystinuria. All three distinct forms of homocystinuria including CBS deficiency, MTHFR deficiency and defective methionine synthase, carry with them a very high risk of premature vascular disease and the only biochemical change that these three defects have in common is an extremely high plasma homocysteine level (200-500 μmol/L) (Rees and Rodgers, 1993). Thus, as proposed by McCully's Homocysteine Theory of Atherosclerosis, it follows that the severe hyperhomocysteinemia (or a homocysteine metabolite) is likely the cause of the premature atherosclerotic changes in homocystinurics and that more modest elevations of homocysteine may contribute to the development of atherosclerosis in the general population (McCully, 1969). It has only been in the last decade that widespread interest in mild hyperhomocysteinemia as a cardiovascular disease risk factor has developed. Over 80 epidemiological and clinical studies have supported homocysteine as a risk factor for atherosclerotic vascular disease as well as arterio and venous thromboembolism (Refsum and Ueland, 1998). A 1995 meta-analysis of 27 studies on homocysteine and cardiovascular disease determined that homocysteine was an independent, graded risk factor for atherosclerotic disease in the coronary, cerebral and peripheral vessels (Boushey et al., 1995). The investigators estimated that homocysteine could be responsible for 10% of the risk of cardiovascular disease in the general population. Additionally, they calculated that an elevation of plasma homocysteine concentration of 5 μmol/L increases the risk of coronary artery disease by as much as an increase of 0.5 mmol/L in the total cholesterol concentration and raises the relative risk of developing coronary artery disease by approximately 60% (CI 40-70%). From the time of that meta-analysis, reports from a number of cross sectional and retrospective case-control studies, of patients suffering from a variety of vascular diseases continue to indicate an association between vascular conditions and homocysteine levels

(Aronow and Ahn, 1997; Aronow and Ahn, 1998; Graham et al., 1997; Malinow et al., 1996; Selhub et al., 1995; Verhoef et al., 1996).

In contrast to the findings of case-control and cross-sectional studies, the results of prospective cohort studies, that investigated the associations between hyperhomocysteinemia and vascular disease, remain inconclusive. Reports from prolonged follow-up of the Physicians' Health Study cohort indicate no significant associations between plasma homocysteine levels and risk of MI, stroke angina or death from CHD (Chasan-Taber et al., 1996; Verhoef et al., 1994; Verhoef et al., 1997b). Neither the Multiple Risk Factor Intervention Trial (MRFIT) nor the (ARIC) cohorts displayed a significant association between hyperhomocysteinemia and stroke or cardiovascular disease risk (Evans et al., 1997; Folsom et al., 1998). Other prospective studies, however, have reported significant associations. Early reports from the Physician's Health Study described a significant relationship between plasma homocysteine levels and risk of MI. The adjusted risk for the highest fifth percentile compared to the bottom ninetieth percentile was 3.1 (Stampfer et al., 1992). Nygard et al (1997) followed patients with angiographically documented coronary artery disease and found a strong graded relationship between homocysteine levels and overall mortality. The mortality ratio was 4.5 for those with homocysteine concentrations of 20 μmol/L compared to those with homocysteine concentrations of 9 μmol/L or less (Nygard et al., 1997). The British United Provident Association (BUPA) prospective study found that the risk of ischemic heart disease was 2.9 times higher among the men in the highest quartile of serum homocysteine levels compared to the men in the lowest quartile for serum homocysteine (95% CI, 1.8-4.7) (Wald et al., 1998). Additionally, it was determined that for each 5 μmol/L increase in serum homocysteine level there was a corresponding 33% increased risk of ischemic heart disease (Wald et al., 1998). Overall, at least ten prospective studies have demonstrated a positive association between homocysteine and atherosclerotic and thrombotic vascular diseases (Arnesen et al., 1995; Bostom et al., 1997b; Moustapha et al., 1998a; Nygard et al., 1997; Perry et al., 1995; Ridker et al., 1999; Stampfer et al., 1992; Taylor et al., 1999; Wald et al., 1998; Whincup et al., 1999). This association appears to be concentration related and remains independent of other established risk factors for vascular disease. However, given the inconsistent finding of prospective studies it would be premature to conclude that hyperhomocysteinemia plays a causal role in the development of disease. Moreover, there remains little evidence that decreasing plasma homocysteine will result in a reduction of cardiovascular disease risk. Hence, the importance of the many ongoing controlled clinical intervention trials attempting to assess

whether homocysteine-lowering therapy will play a role in the primary and/or secondary prevention of cardiovascular events.

Vitamins, Homocysteine and Vascular Disease

As vitamins play an essential role in the metabolism of homocysteine, it is important to identify the relationship, if any, between vitamins, homocysteine and cardiovascular disease. A case control study by Robinson et al. (1998) found that folate deficiency was more common in cases of arterial disease than in control subjects. It was also noted that, for control subjects, plasma vitamin B_6 levels below the 20th percentile were associated with increased long-term risk of vascular disease and that this relationship was independent of homocysteine levels. Results from the prospective Nutrition Canada Survey indicated that over a 20-year period the risk of fatal coronary artery disease is associated with low serum folate levels (Morrison et al., 1996). In the elderly Framingham cohort, folate and PLP (but not vitamin B_{12}) status were linked to carotid artery stenosis prevalence (Selhub, 1997). Statistical adjustment of vitamin levels for homocysteine levels diminished the odds ratio between stenosis and vitamin status, thus, it appears that much of the link between vitamin status and stenosis was due to the influence of folate and vitamin B_6 on homocysteine levels (Selhub, 1997). Pancharuniti et al (1994) found similar results in a case-control study. They reported an association between plasma folate concentration and cardiovascular disease risk; once more the risk was greatly diminished after adjustment for plasma homocysteine concentrations (Pancharuniti et al., 1994). Users of multivitamins have been reported to have a lower incidence of vascular disease and lower homocysteine levels than nonusers (Graham et al., 1997; Rimm et al., 1998). Interestingly, most reports indicate that low vitamin B_{12} levels are not associated with increasing cardiovascular disease risk; in fact, vitamin B_{12} concentrations have been found to be directly related to risk of cardiovascular disease (Folsom et al., 1998; Pancharuniti et al., 1994). It has been suggested that this direct link may be due to the association between vitamin B_{12} foods high in saturated fat (Pancharuniti et al., 1994). Taken as a whole, the evidence does support an inverse association between folate status and vascular disease that appears to be a result of the regulatory effect of folate on homocysteine levels. However, the relationships of vitamin B_6 and vitamin B_{12} to cardiovascular disease risk homocysteine levels are much less clear.

Treatment of Hyperhomocysteinemia

Mildly elevated homocysteine levels can be reduced with dietary modifications and/or vitamin therapy. Dietary modification designed to raise folate intake (to a total folate content of 560 μg/d) by increasing the consumption of vegetables and citrus fruits reduced homocysteine concentrations up to 17% in healthy subjects (Brouwer et al., 1999). The Food and Nutrition Board of the Institute of Medicine has recommended that the upper limit of folic acid supplementation should be 1 mg/d as higher doses may mask the symptoms of vitamin B_{12} deficiency (Institute of Medicine, 1998). Supplementation of vitamin B_6, vitamin B_{12} and folic acid have all been used, either alone, or in various combinations, for the treatment of mild hyperhomocysteinemia. In most cases the minimal effective daily dose of folic acid for achieving maximum homocysteine lowering effect is 0.4 mg with no additional benefit of doses exceeding this amount (Boushey et al., 1995; Brattstrom et al., 1998; Malinow et al., 1998; Malinow et al., 1997). Supplementation with vitamin B_{12} alone is generally effective at decreasing homocysteine levels in cases of vitamin B_{12} deficiency but it is less effective than the supplementation of vitamin B_{12} in combination with folic acid (Brattstrom et al., 1988; Naurath et al., 1995; Savage et al., 1994; Stabler et al., 1997). Supplementation with pyridoxine in doses up to 300 mg/d does not appear to lower fasting homocysteine levels in healthy subjects or patients with vascular disease (Brattstrom, 1996; Dierkes et al., 1998; Franken et al., 1994). However, post-methionine load hyperhomocysteinemia can be reduced by pyridoxine supplementation at the level of 50-250 mg/d (Bostom et al., 1997; Franken et al., 1994). A meta-analysis of 12 randomized, controlled trials that assessed the effectiveness of folic acid, vitamin B_{12} and vitamin B_6 supplementation on blood homocysteine levels found that the proportional and absolute reductions in homocysteine produced by folic acid were greater at higher pretreatment homocysteine concentrations and at lower pretreatment blood folate levels (Brattstrom et al., 1998). Furthermore, it was determined that after standardization of plasma homocysteine and folate levels to the approximate average value for western populations (12 μmol/L and 12 nmol/L respectively), dietary folic acid supplementation (0.5-5.0 mg daily) reduced blood homocysteine concentrations by 25%. Addition of vitamin B_{12} (mean 0.5 mg daily) produced a further homocysteine reduction of 7% but vitamin B_6 (mean 16.5 mg daily) did not have a significant additional homocysteine lowering effect. Interestingly, it was recently reported that treatment of healthy siblings of patients with premature atherothrombotic disease with 5 mg folic acid and 250 mg vitamin B_6 daily for 2 years was associated with both a decline in fasting and post-methionine load plasma homocysteine concentrations and a decreased occurrence of abnormal exercise electrocardiography tests

(Vermeulen et al., 2000). This study suggests that vitamin treatment designed to lower homocysteine levels also modifies the course of atherosclerotic disease.

The American Heart Association does not yet recommend population-wide screening for hyperhomocysteinemia. Instead, they advise emphasizing the importance of meeting the Recommended Dietary Allowance for folate, vitamin B_6 and vitamin B_{12} by intake of vegetables, fruits, legumes, meats, fish and fortified grains and cereals (Malinow et al., 1999). The 1991 Recommended Nutrient Intake (RNI) for folate (200μg/day) has been found to be inadequate to maintain folate status or low plasma homocysteine concentrations in most subjects (Jacob et al., 1994). However, the new Recommended Daily Allowance for folate intake for adult men and women will be 400 folate equivalents per day (Institute of Medicine, 1998). Although this recommendation is not designed to minimize plasma homocysteine concentrations, it should be more effective than the current RNI toward maintaining plasma homocysteine at stable low levels. Screening for hyperhomocysteinemia is suggested for certain high-risk patients, and if hyperhomocysteinemia is present the daily use of supplemental vitamin B_6 (2 mg), vitamin B_{12} (6 μg) and folic acid (0.4 mg) is recommended, by the American Heart Association, as an experimental treatment (Malinow et al., 1999).

The recent policy of folic acid fortification of flours and cereals with 140 μg folic acid/100 g raised the plasma folate concentrations of the Framingham Offspring Study cohort from 11 to 23 nmol/L (Jacques et al., 1999). At the same time their plasma homocysteine concentrations decreased from 10.1 to 9.4 μmol/L, and the prevalence of high homocysteine concentrations (> 13 μmol/L) decreased from 18.7 to 9.8% (Jacques et al., 1999). It is not yet known whether this level of fortification will result in a reduction in the incidence of cardiovascular disease.

Pathogenesis of hyperhomocysteinemia

Many mechanisms have been proposed to explain the atherogenic propensity associated with hyperhomocysteinemia. These mechanisms include increased vascular smooth muscle cell proliferation (Frauscher et al., 1995; Lubec et al., 1996; Stamler and Slivka, 1996; Tsai et al., 1994) generation of reactive oxygen species (Heinecke et al., 1993; Hultberg et al., 1997; Kanani et al., 1999; Ueland et al., 1996; Voutilainen et al., 1999), increased platelet adherence and alteration of blood clotting factors (Lentz and Sadler, 1991; Nishinaga and Shimada, 1994; Rodgers and Conn, 1990; Rodgers and Kane, 1986). A hypothesized sequence of events begins

with homocysteine induced endothelial dysfunction followed by platelet activation and thrombus formation (Eikelboom et al., 1999). In a recent review paper Welch and Loscalzo (1998) proposed a potentially unifying hypothesis for the vascular damage associated with hyperhomocysteinemia. They suggested that it is the generation of potent oxygen free radicals by homocysteine that leads to the proliferation of smooth muscle cells, alterations in structure and function of the vascular endothelium, increased thrombogenic activity and ultimately atherothrombosis (Welch and Loscalzo, 1998).

The promotion of free radical mediated damage by homocysteine lacks firm experimental backing despite biological plausibility. When added to plasma, homocysteine is rapidly auto-oxidized to homocystine, homocysteine-mixed disulfides and homocysteine thiolactone (Andersson et al., 1995; Stamler et al., 1993). The oxidation of the sulphydryl group can generate hydrogen peroxide, superoxide and hydroxyl radicals, especially in the presence of ferric or cupric ions (Bellamy and McDowell, 1997). Homocysteine is cytotoxic to cultured endothelial cells in the presence of cupric ions, but this effect can be reversed by the addition of catylase, thus suggesting that the cytotoxicity is mediated by hydrogen peroxide (Starkebaum and Harlan, 1986). Homocysteine also initiates superoxide-mediated oxidation of lipoproteins *in vitro*, however, the same effect can be obtained with cysteine, indicating a general thiol effect (Heinecke et al., 1987). *In vivo* studies have had mixed results regarding the susceptibility of plasma lipids to oxidation in subjects with hyperhomocysteinemia (Cordoba-Porras et al., 1996; Voutilainen et al., 1999). It is possible that free radical mechanisms other than lipid peroxidation could contribute the proposed atherogenicity of homocysteine. For example, nitric oxide is rendered biologically inactive in the presence of reactive oxygen species (Berman and Martin, 1993). Clearly, homocysteine can facilitate the production of free radicals, but whether this is biologically important remains to be determined.

The procoagulatory effect of hyperhomocysteinemia has been contributed to a number of factors. Homocysteine reduces the activation of protein C, which is important for fibrinolysis (Lentz and Sadler, 1991; Rodgers and Conn, 1990). Homocysteine has also been shown to suppress thrombomodulin and heparin sulfate activity, increase Factor V activity and increase thromboxane A_2 production (Graeber et al., 1982; Lentz and Sadler, 1991; Nishinaga and Shimada, 1994; Rodgers and Kane, 1986). Most of the procoagulatory effects of homocysteine have only been demonstrated *in vitro* using higher than physiological concentrations of

homocysteine, thus, the true *in vivo* effects of mild hyperhomocysteinemia on coagulation are not known.

There is a burgeoning body of evidence to support the hypothesized mechanisms of vascular damage by homocysteine and most indicate that endothelial damage plays an important role. Hyperhomocysteinemia is associated with impaired endothelia function in humans as measured by endothelium-dependent flow mediated dilation (Woo et al., 1997). Experimental hyperhomocysteinemia, produced by methionine load, leads to substantial decreases in flow-mediated endothelial dilation in monkeys, rats and humans (Kanani et al., 1999; Lentz et al., 1996; Ungvari et al., 1999). Flow mediated dilation is mainly regulated by endothelium-derived relaxing factor, now known as nitric oxide (NO) (Kelly et al., 1996). *In vitro* evidence indicates that normal endothelial cells detoxify homocysteine by releasing NO; the two compounds rapidly react to form S-nitroso-homocysteine (Stamler et al., 1993). The S-nitrosation of homocysteine decreases its pathogenicity by inhibiting the sulphydryl-dependent generation of hydrogen peroxide; as well S-nitroso-homocysteine acts as an antiplatelet agent and vasodilator (Loscalzo, 1996). Initially, endothelial cells respond to homocysteine by increasing the production NO and thus S-nitroso-homocysteine, however, the protective action of S-nitroso-homocysteine may eventually be overcome when homocysteine-induced, oxidative stress renders NO production and/or activity insufficient to balance homocysteine levels (Bellamy and McDowell, 1997; Loscalzo, 1996; Stamler et al., 1993). Thus, prolonged exposure to homocysteine leads to decreased production of NO and unopposed homocysteine-mediated oxidative injury may ensue (Stamler et al., 1993). Interestingly, the impairments in endothelial function that occur during experimentally-induced, mild hyperhomocysteinemia can be prevented or reversed by the oral administration of the antioxidant vitamin ascorbic acid, further supporting the hypothesis that the adverse effects of hyperhomocysteinemia are mediated through oxidative stress mechanisms (Chambers et al., 1999; Kanani et al., 1999).

Reference ranges for homocysteine

There is widespread disagreement regarding the establishment of safe reference ranges for plasma homocysteine. The risk for vascular disease begins to increase with homocysteine values of 10.5-11.7 μmol/L (Stampfer et al., 1992). It has been estimated that up to 40% of the population is not consuming enough folate to keep homocysteine levels low (Stampfer and Willett, 1993). Thus, it has been suggested that reference ranges be determined in a population with a vitamin status

confirmed to be adequate (Rasmussen et al., 1996; Rossi et al., 1999; Ubbink et al., 1995). Studies carried out in folate-replete subjects have resulted in reference ranges for homocysteine that vary from 5.0-12.1 μmol/L to 8.3-13.7 μmol/L for men and 4.4-9.8 μmol/L to 6.5-11.9 μmol/L in women (Rasmussen et al., 1996; Rossi et al., 1999). When reference ranges for homocysteine are calculated without consideration for vitamin status the resulting upper limit for normal plasma homocysteine concentration is much higher (15-16 μmol/L) and is well above the level where increased cardiovascular disease risk begins (Rasmussen et al., 1996; Stampfer et al., 1992; Ueland et al., 1993). There has been no agreement reached regarding which values are the most appropriate values for normal homocysteine reference ranges, and, as evidence supporting hyperhomocysteinemia as a risk factor for cardiovascular disease mounts, the importance of establishing a normal reference range increases.

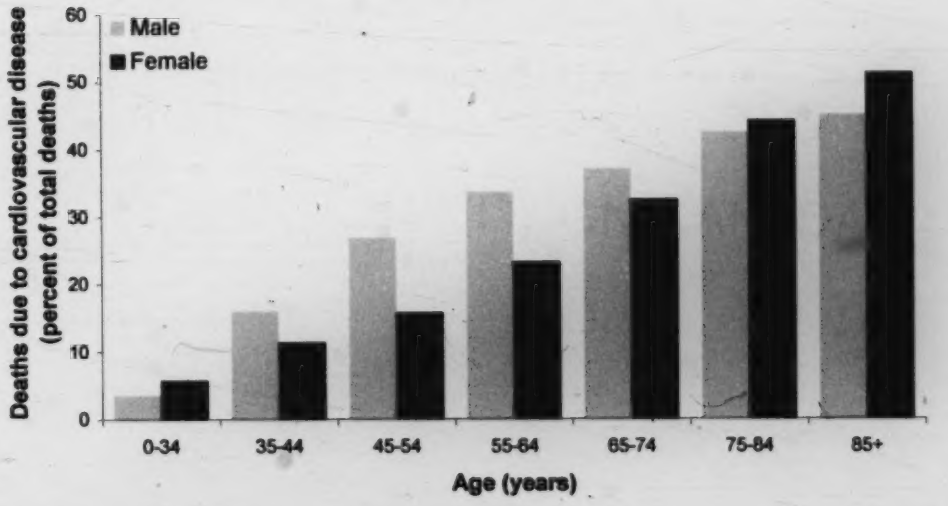
Interactions between cholesterol and homocysteine

Although homocysteine is considered an independent risk factor for cardiovascular disease, there is some evidence supporting an interaction between homocysteine and cholesterol. Epidemiological evidence indicates a positive correlation between plasma homocysteine and cholesterol concentrations in subjects with hyperhomocysteinemia (Olszewski et al., 1989). *In vitro* evidence indicates that homocysteine has the ability to stimulate the production and secretion of cholesterol in hepatic cell cultures, an effect that appears to be mediated by the enhancement of HMG-CoA reductase activity (Lynn et al., 1998). It is, however, important to note that the concentration of homocysteine used to study this effect was approximately two hundred times higher than normal physiological levels (Lynn et al., 1998). Zulli et al (1998) reported a synergistic effect of homocysteine and cholesterol feeding on raising the plasma levels of homocysteine, cholesterol and triglycerides in rats. The combined effect of homocysteine and cholesterol feeding was much higher than the influence of feeding cholesterol or methionine alone, possibly indicating the importance of dietary factors, other than vitamins, on the outcome of hyperhomocysteinemia (Zulli et al., 1998a). Furthermore, the combination of methionine and cholesterol feeding in rats has been found to act synergistically in promoting the disintegration of the elastic lamina in the arterial wall when compared to the feeding of either agent alone (Zulli et al., 1998b). Although preliminary, these studies indicate that it may be important to investigate the combined effects of cholesterol and homocysteine on cardiovascular disease as well as each agent alone (Zulli et al., 1998b). More work is needed to determine whether hyperhomocysteinemia can lead to the development of elevated plasma cholesterol or visa versa.

Age-related issues in nutrition

The proportion of the Canadian population that is older than 65 is increasing rapidly. It has been predicted that by the year 2011, approximately 14% of the population will be at least 65 years of age or older (Statistics Canada, 1999). Thus, there is an increasing need for nutrition information concerning the preservation of health and quality of life for older individuals. This includes knowledge regarding the importance of nutrition in the development, susceptibility, outcome and treatment of chronic diseases. The leading cause of death and disability in adults is cardiovascular disease (Statistics Canada, 1999). Although the development of atherosclerosis begins in childhood there is a substantial increase in the incidence of cardiovascular disease around 45 years of age for men and around 55 years of age for women (Heart and Stroke Foundation of Canada, 1997). In both sexes, the percentage of deaths due to cardiovascular disease increases dramatically in the older age groups (Figure 3) (Statistics Canada, 1999). It is likely that the societal impact of cardiovascular disease will increase as the proportion of the older population expands. Thus, the identification and treatment of modifiable cardiovascular disease risk factors in this age group may become increasingly important.

Figure 3: Percentage of deaths due to cardiovascular disease, specific to age group and gender, Canada

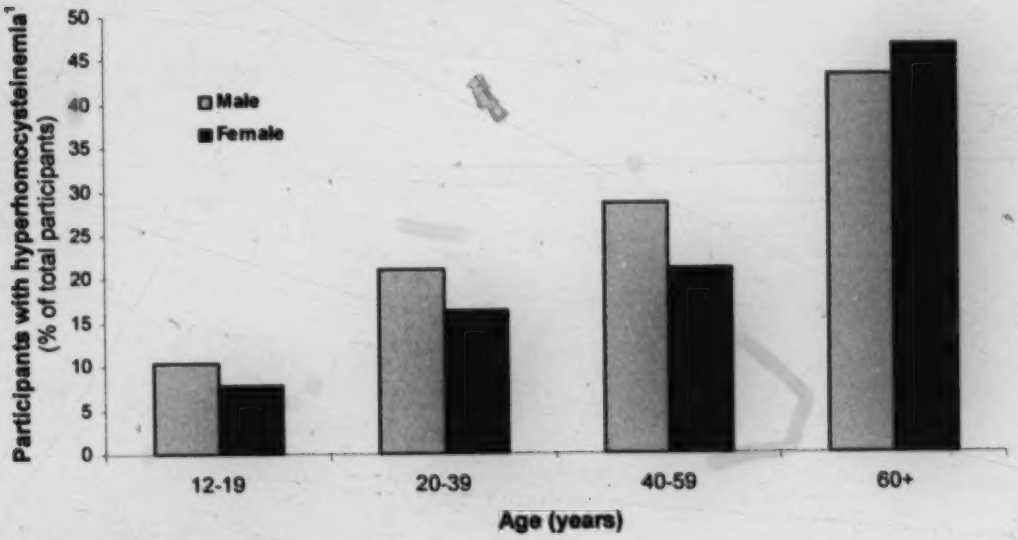


1995 (adapted from (Statistics Canada, 1999)).

The precise reasons for the age-related increase in cardiovascular disease remain to be determined. But, before the associations between aging and the development of disease are fully understood a better comprehension of normal aging, including age-related changes in cardiovascular disease risk factors, is essential.

Hyperhomocysteinemia is becoming increasingly accepted as an independent risk factor for cardiovascular disease. Many studies have identified a positive relationship between age and plasma homocysteine levels in both healthy subjects and those with cardiovascular disease (Andersson et al., 1992a; Brattstrom et al., 1994; Brattstrom et al., 1992; Jacques et al., 1999; Nygard et al., 1995; Robinson et al., 1995; Rossi et al., 1999; Selhub et al., 1993). Thus, as homocysteine concentrations increase with age, so does the prevalence of hyperhomocysteinemia (Figure 4) (Selhub, 1999). There are a variety of factors that may contribute to the age-related increase in homocysteine levels, but the specific causes have not been established. The potential contributing factors include impaired activity of cystathionine-β-synthase, declining renal function, and poor intake, absorption and/or utilization of B-vitamins (Koehler et al., 1996; Nordstrom and Kjellstrom, 1998; Norlund et al., 1998; Selhub et al., 1993). Regardless of its cause, the age-related increase in homocysteine concentrations, coupled with the suspected role of homocysteine in vascular disease, indicate that hyperhomocysteinemia may be placing a substantial proportion of the elderly population at increased risk for vascular disease.

Figure 4: Prevalence of hyperhomocysteinemia by age and sex



Adapted from Selhub et al. 1999

[1]Defined as plasma homocysteine > 11.4μmol/L for male and >10.4 μmol/L for female participants

Declining renal function is a common problem in the elderly. It has been suggested that this decline may be responsible for the increase in homocysteine concentrations associated with age, however, studies designed to explore this hypothesis are limited (Brattstrom et al., 1994; Norlund et al., 1998). Norlund et al (1998) found that homocysteine, serum creatinine and plasma cystatin C increased with age in a group of healthy Swedish subjects age 20-88 years. Plasma cystatin C is a low molecular weight marker for glomerular filtration rate that is not related to the formation of creatinine and, unlike creatinine, is not influenced by muscle mass (Norlund et al., 1998). Using linear regression, the investigators demonstrated that plasma cystatin C had a higher predictive value for homocysteine concentrations than other factors, such as age or serum creatinine (Norlund et al., 1998). The combination of plasma cystatin C and age slightly increased the explanatory power, thus indicating that some of the increase in homocysteine with age could be partly explained by age-related decline in renal function.

Another alternative explanation for the age-related increase in plasma homocysteine levels arose from *in vitro* investigations. Studies using cultured human fibroblasts and lymphocytes indicate that cystathionine-β-synthase activity decreases with age (Gartler et al., 1981; Nordstrom and Kjellstrom, 1992). Whether this decrease plays a role in the age-related increase in homocysteine concentrations is yet to be determined and merits further investigation.

The hypothesized mechanism for the age-related increase in homocysteine levels that has received the most attention is compromised vitamin status. Much of the focus is derived from the fact that subclinical water-soluble vitamin deficiencies are not uncommon in the elderly population, even in those meeting the recommended intake levels (Suter and Russell, 1987). Vitamin requirements for people older than 65 years of age are largely extrapolated from data derived from younger adults (Chernoff, 1995). Thus, deficiencies may occur in people who meet, but do not exceed, the recommended vitamin intake levels because the absorption or utilization of vitamins may be compromised by increasing metabolic demands, polypharmacy, or overuse of single nutrient supplements (Chernoff, 1995). In the face of injury or illness the marginal tissue stores may not be able to compensate for rapid depletion and recovery from the illness may be compromised (Baik and Russell, 1999; Chernoff, 1995). This underscores the importance of defining adequate vitamin nutrition in health maintenance in an aging population, and, in the case of age-related hyperhomocysteinemia, adequate vitamin nutrition may be an important factor in disease prevention.

Two of the most common vitamin deficiencies in the elderly are vitamin B_{12} and folate (Andres and Hallfrisch, 1989; Quinn and Basu, 1996). The key nutrient of concern in hyperhomocysteinemia is usually folate, however, that is not necessarily the case in older individuals. A significant portion of the elderly population (5-25%) is thought to suffer from marginal or deficient vitamin B_{12} status (Baik and Russell, 1999; Lindenbaum et al., 1994; Quinn and Basu, 1996; Stabler et al., 1997; van Asselt et al., 1998). Vitamin B_6 is also known to decline towards deficient levels with advancing age. Up to 22% of the elderly population has been found to have low plasma PLP levels (van der Wielen et al., 1996). Finally, the prevalence of folate deficiency in the elderly is approximately 2-20%, depending on the diagnostic criteria used (Quinn and Basu, 1996; Sauberlich et al., 1987).

Plasma folate and vitamin B_{12}, but not vitamin B_6, levels were the major determinants of plasma homocysteine levels in an elderly Swedish cohort (Brattstrom et al., 1994). A strong association between homocysteine and dietary intake of vitamins B_6 and folate, but not vitamin B_{12}, was found among the elderly subjects in the Framingham study (Selhub et al., 1993). In the same cohort, higher plasma levels of vitamin B_6, vitamin B_{12} and folate were all linked to lower plasma homocysteine levels. However, serum MMA, as an indicator of vitamin B_{12} status, entered the model before serum folate and explained more of the overall variance in serum homocysteine (Selhub et al., 1993). Nevertheless, the association between age and homocysteine concentrations persisted after adjustment for plasma B vitamin levels (Selhub et al., 1993). In a study conducted by Joosten et al. (1993), 14.4% of the variation in plasma homocysteine in elderly people was explained by plasma folate concentration, while only 4.4% of the variation in plasma homocysteine concentrations could be explained by variations in plasma folate concentration in younger people. The inverse relationship between plasma folate and homocysteine levels was stronger in elderly subjects compared to young subjects. Examination of homocysteine concentrations in participants of the Third National Health and Nutrition Examination Survey (NHANES III) revealed a strong inverse association between plasma homocysteine and folate levels and a slightly weaker but still significant relationship between plasma homocysteine and vitamin B_{12} levels at all ages (Selhub, 1999). The total population-attributable risk for high homocysteine concentrations, associated with low concentrations of either vitamin B_{12} or folate, decreased from approximately 75% in participants younger than 40 years of age to approximately 31% for participants 60 years of age or older (Selhub, 1999). Thus, it seems that age may exert an influence on the associations between hyperhomocysteinemia and plasma vitamin concentrations. Investigators attributed this finding to the decreased contribution of low folate concentrations, but not low vitamin B_{12} concentrations, to high homocysteine levels in the elderly (Selhub, 1999). It was also suggested that the contribution of low vitamin B_{12} to hyperhomocysteinemia in this age group might be a result of the increased prevalence of low vitamin B_{12} concentrations with age, along with the accrual with age of other risk factors for high homocysteine, such as declining renal function, and probably estrogen status in women (Selhub, 1999). Herrmann et al (1999) observed an age-related increase in plasma homocysteine levels in a group of subjects, ranging in age from 65-104 yrs, however, no correlation between the homocysteine elevation with age and serum vitamin levels was present.

Low folate and vitamin B_{12} levels have been implicated in cognitive impairment, hearing loss and vascular disease in elderly people (Ebly et al., 1998; Kalmijn et al., 1999). Plasma homocysteine levels in these studies were also linked to these ailments; however, it is unclear whether or not the age-related increase in homocysteine plays a role in the development of many age-associated disorders. It has been hypothesized that prolonged elevations of plasma homocysteine result in the accumulation of various homocysteine-adducts, the formation of which is dependent on time and homocysteine concentration (Krumdieck and Prince, 2000). Over time, the function of the homocysteinylated proteins would be diminished or completely lost, cumulative damage would occur and eventually clinical manifestations would become evident (Krumdieck and Prince, 2000).

All of the vitamins involved in homocysteine metabolism have been implicated in the age-related increase in homocysteine levels, but deficiency of any single vitamin, or combination of vitamins, does not appear to completely explain this increase. There is some indication that low levels of vitamin B_{12} may be a more important contributing factor for hyperhomocysteinemia in the elderly than in younger individuals, but the contribution of low folate status to hyperhomocysteinemia in the elderly cannot be ignored.

Impaired absorption of vitamin B_{12} is common in the elderly population due to the existence of an age-related increase in the incidence of pernicious anemia and atrophic gastritis (Russell, 1997). Natural dietary vitamin B_{12} is limited to animal sources and it is present in these sources bound to protein (Herbert, 1988). The first step in vitamin B_{12} absorption is the cleavage of the vitamin-protein bond (Carmel, 1995). This dissociation is accomplished though the action of gastric HCL and pepsin. Atrophic gastritis affects approximately 20-30% of people older than 60 yrs of age and is thought to be the main cause of vitamin B_{12} deficiency in the elderly (Russell, 1997). A smaller proportion of older adults, approximately 2%, are deficient in vitamin B_{12} as a result of pernicious anemia (Carmel, 1997). Free vitamin B_{12} in the stomach binds to R-protein, secreted by the salivary glands and gastric mucosa (Carmel, 1995). Within the alkaline environment of the small intestine, pancreatic enzymes degrade the R-protein, freeing vitamin B_{12}. The resulting free vitamin B_{12} binds to intrinsic factor, which is secreted from the parietal cells of the stomach after stimulation by food (Baik and Russell, 1999). In the terminal ileum the vitamin B_{12} - intrinsic factor complex attaches to specific membrane receptors on the enterocytes and is absorbed by phagocytosis (Baik and Russell, 1999). Pernicious anemia is characterized by the inability to produce intrinsic factor. Therefore, it impairs the absorption of both protein-bound

and free vitamin B_{12}. Atrophic gastritis is characterized by hypochlorhydria, impaired intrinsic factor excretion and low pepsin production (Baik and Russell, 1999). The hypochlorhydria and low acid-pepsin production impair protein-bound vitamin B_{12} absorption (Baik and Russell, 1999). Despite the slight impairment of intrinsic factor production that occurs in atrophic gastritis, there is sufficient intrinsic factor present to support the normal absorption of crystalline vitamin B_{12} (Iber et al., 1994). The American National Academy of Sciences Institute of Medicine recommends that adults older than 50 years should obtains 2.4 μg of vitamin B_{12} per day, primarily in the highly absorbable synthetic form present in fortified foods and supplements (Institute of Medicine Subcommittee on Folate, 1998).

In January 1998, folic acid fortification of the food supply was undertaken to reduce the risk of neural tube defects. There is also speculation that it may have an additional benefit on vascular disease because of the relationship between inadequate folate intake and higher circulating plasma homocysteine concentrations (Boushey et al., 1995; Tucker et al., 1996). However there is a reason to be concerned about the effect of folate fortification on older individuals. The prevalence of vitamin B_{12} deficiency in the elderly population has been estimated to be between eight and twenty-five percent and the deficiency of this vitamin has been associated with impaired cognitive function, dementia and neuropsychiatric disorders (Baik and Russell, 1999; Lindenbaum et al., 1994; Quinn and Basu, 1996; Stabler et al., 1997; van Asselt et al., 1998). Unfortunately, these symptoms are often present in the absence of normal indicators of vitamin B_{12} deficiency, such as low serum vitamin B_{12} levels, macrocytic anemia or abnormal Shilling tests but in the presence of elevated homocysteine or MMA (Baik and Russell, 1999; Riggs et al., 1996). The possibility exists that increasing dietary folate intake could mask the hematologic symptoms associated with vitamin B_{12} deficiency and a delay in the correct diagnosis could result in an increase in the frequency and severity of neuropsychiatric complications (Baik and Russell, 1999). Thus, it has been recommended that more attention be given to the screening and treatment of vitamin B_{12} deficiency in older people (Stabler et al., 1997). Furthermore, vitamin B_{12} deficiency should be ruled out before starting folate supplementation in an elderly individual. It has been suggested that folate supplements should contain cyanocobalamin at 100-200 times the required level as approximately 1-2% of the oral dose will be absorbed by passive diffusion regardless of the presence of pernicious anemia or atrophic gastritis (Stabler et al., 1997).

Criteria for aging research

Criteria for animal models for aging research must be considered before initiating research on aging (Table 2). Proper study design requires knowledge of the median length of life and maximum life span of the animal model to be used (Masoro, 1987). A species with a short life span enables investigators to conduct multiple aging studies during the course of their careers. As well, it is easier to conduct longitudinal research, under well-defined conditions, in animals with shorter life spans. To determine the normal physiologic characteristics of aging it is essential to keep the animals free of infectious diseases, as these diseases can decrease the life span and increase the variability in the model (Andre et al., 1996; Masoro, 1987; Smith and Breslow, 1997). It is also important to be familiar with the genetic characteristics of the animal model used, thus, the use of inbred strains is common. The lack of genetic heterogeneity produced by this practice means that there is less variability between animals from different suppliers (Fekete, 1993; Masoro, 1987). However, the practice of inbreeding creates strains that often suffer from a single major disease process that may complicate the interpretation of the research results. Thus, proper interpretation of aging research demands that the investigator be familiar with the pathologic changes that commonly occur with age in the animal model under consideration.

Table 2: Ideal criteria for the use of animal models in aging research[1]

1.	Available life span data
2.	Short-life span
3.	Capacity to maintain animals free from infectious diseases
4.	Knowledge of genetic characteristics
5.	Capacity to maintain defined environmental characteristics
6.	Available data regarding pathological changes associated with aging
7.	Animals easily available
8.	Reasonable cost of purchase and maintenance

[1]modified from (Masoro, 1987)

The maintenance of defined environmental conditions is another important step in limiting variability in the model and isolating the normal aging process. Aside from maintaining a pathogen-free environment, the most important consideration is nutrition. Not only is it important that all animals receive the same type of food and water, but the amount of food must be similar as well. For example, when male Fisher 344 rats are allowed unlimited access to food, progressive renal nephropathy and renal failure occurs in more than 50% of the animals (Committee on Animal Models for Research on Aging, 1981). Alternatively, rats restricted to 60% of ad libitum intake rarely develop nephropathy at all (Committee on Animal Models for Research on Aging, 1981). When obtaining animals from an outside supplier for the purpose of aging research it is essential that accurate information be available to the investigator regarding the lifetime environment of the animal including dietary history and infectious disease monitoring data (Committee on Animal Models for Research on Aging, 1981). The cost of obtaining and maintaining such animals continues to be a barrier to aging research.

The animal model selected for the present study is the male Fisher 344 rat. These rats are commonly used for aging research; thus, a wide variety of information is available regarding the normal age-related changes that take place in this species. The National Institute of Aging maintains colonies of Fisher 344 rats specifically for use in aging research. Accordingly, detailed data regarding the lifetime environment of the rats is made available to investigators utilizing these animals. When these rats are raised 2-3 per cage in a pathogen-free environment and are provided with free access to food and water, they have a maximum lifespan of approximately 26 months (Masoro, 1980). Thus, in an effort to examine the normal age related changes that take place from mid-life to old age, rats aged 12 to 24 months were used in the present study.

CHAPTER 2: RATIONALE

It has been known for over 25 years that severely elevated plasma homocysteine levels, induced by inherited enzymatic disorders of homocysteine metabolism, are closely linked to atherosclerotic vascular disease. Recently, it has become clear that even mild to moderate hyperhomocysteinemia is an independent risk factor for both atherosclerosis and atherothrombosis. The susceptibility to these vascular diseases increases throughout the aging process, making vascular disease the largest cause of mortality among elderly individuals. Homocysteine may play a role in this age-related increase in cardiovascular disease, as there is increasing evidence that hyperhomocysteinemia is common in the elderly population. The possible causes of this hyperhomocysteinemia are multiple and include impaired vitamin status, declining renal function and lifestyle influences. Vitamins are of particular interest in the elderly population as they commonly suffer from marginal nutrient deficiencies. Normal homocysteine metabolism depends on the vitamin B_6-dependent trans-sulfuration of homocysteine to cysteine, and the folate and vitamin B_{12}-dependent transmethylation of homocysteine to methionine. Low folate status appears to be the most important contributing factor to hyperhomocysteinemia in the general population, but this is not necessarily true in elderly individuals as the importance of vitamin B_{12} as a determinant of homocysteine levels appears to increase with age. Thus, there are a variety of factors that may contribute to the age-related increase in homocysteine concentrations but the specific causes have not been established. Additionally, there is some indication that there is a relationship between plasma homocysteine and cholesterol levels. Both homocysteine and cholesterol are considered to be risk factors for cardiovascular disease. Limited work has been done to characterize the interaction between them, and even less is known about them in relation to the aging process. It is difficult to identify normal age-related changes and the mechanisms causing them in humans, as there are many physiological, psychological and social barriers to conducting aging research on the human population. To determine the effects of the aging process in the absence of disease, polypharmacy and interfering social factors, aging research is conducted in appropriately controlled animal models. Very little is known about the influence of age on homocysteine metabolism in animal models as most of the homocysteine research in animals occurs while the animals are very young. In the case of rodents, homocysteine research often takes place in young animals that are still growing.

Hypotheses and overall objectives

The following hypothesis were made:

1. Plasma homocysteine concentrations increase with age as a result of declining folate status.
2. The age related increase in homocysteine concentrations will be accompanied by an increase in lipid levels.
3. Improving folate status can attenuate the age-related increase in homocysteine.

The hypotheses were tested in Fisher 344 rats with the following objectives:

1. To determine the influence of age on the plasma homocysteine levels, and the B-vitamins involved in its metabolism (vitamin B_6, vitamin B_{12} and folate) in an aging rat model.

2. To determine whether there is a relationship between age-related hyperhomocysteinemia, the status of the vitamins involved in homocysteine metabolism, and the concentrations of selected plasma and liver lipids.

3. To determine whether improving folate status attenuates the age-associated increase in plasma homocysteine concentrations.

CHAPTER 3: MATERIALS AND METHODS

Animals and Diets

Male Fisher 344 rats aged 12, 18 and 24 months were obtained from the National Institute of Aging (NIA) colonies (Harlan Spraque Dawley Inc. contractor, Indianapolis, IN). These rats were housed 2-3 per cage in plastic shoebox cages and maintained on a 12-hour light/dark cycle in a temperature and humidity controlled room. Animals were allowed free access to a non-purified diet (NIH-07, Zeigler Bros., Ins. Gardeners, PA) and water. Body weights were recorded throughout the study. Composition of the diet is given in Table 3 and the analysis of vitamin/mineral content of the diet, following the addition of the vitamin and mineral premixes, is given in Table 4.

Experiment 1: After an acclimatization period of two weeks, the rats, aged 12, 18 and 24 months, were transferred to wire mesh bottom, stainless steel metabolic cages and housed individually for one week (Figure5). Food intake of these animals was recorded on a daily basis. Following an overnight fast, the animals were anesthetized (3-5% halothane, 1.5-3.0 mL/min) and blood was taken by cardiac puncture, then transferred immediately into chilled Vaccutainers containing either sodium heparin or EDTA. Vaccutainers were placed immediately on ice and protected from light until plasma was separated by centrifugation. Livers were removed, excised, and weighed then frozen in liquid nitrogen. Plasma and liver samples were protected from light and stored at -70°C until analysis.

Figure 5: Protocol of Experiment 1

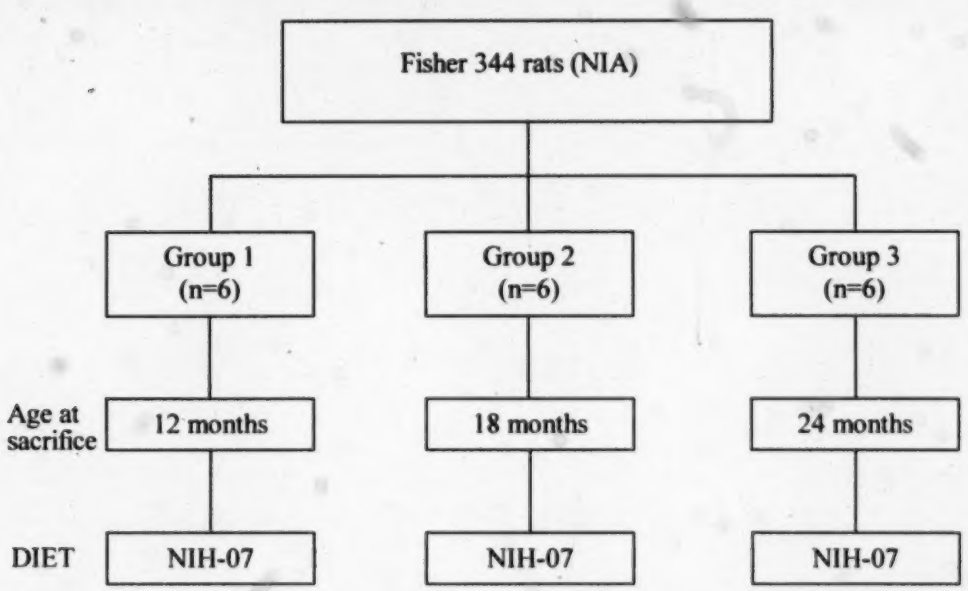


Table 3. Composition of NIH-07 diet[1]

Ingredient	g/kg diet
Dried skim milk	50
Fish meal	100
Soybean meal	120
Alfalfa meal	40
Corn gluten meal	30
Ground corn	245
Ground wheat	230
Wheat middling	100
Brewers dried yeast	20
Dry molasses	15
Soybean oil	25
Sodium chloride	5
Calcium phosphate	12.5
Pre-mixes[2]	2.5

[1]Supplied by Zeigler Bros., Inc. Gardeners. PA. NIH-07 diet contains (g/kg diet): protein (215); carbohydrate (514); fat (52); fiber (32); water (125) [composition provided by supplier for lot number used].

[2]Pre-mixes provided (per kg diet): thiamin (11 mg); riboflavin (3.7 mg); niacin (33 mg); pyridoxine (1.9 mg); vitamin B_{12} (4 μg); folic acid (4 mg); d-pantothenic acid (20 mg); biotin (0.15 mg); choline (0.6g); vitamin A (6050 IU); vitamin D_3 (5060 IU); vitamin K (3.1 mg); tocopheryl acetate (22 IU); cobalt (44 mg); copper (4.4 mg); iron (132 mg); manganese (6 mg); zinc (18 mg); iodine (1.5 mg) [composition provided by supplier for lot number used].

Table 4. Actual vitamin and mineral content of NIH-07 diet following the addition of vitamin and mineral premixes[1]

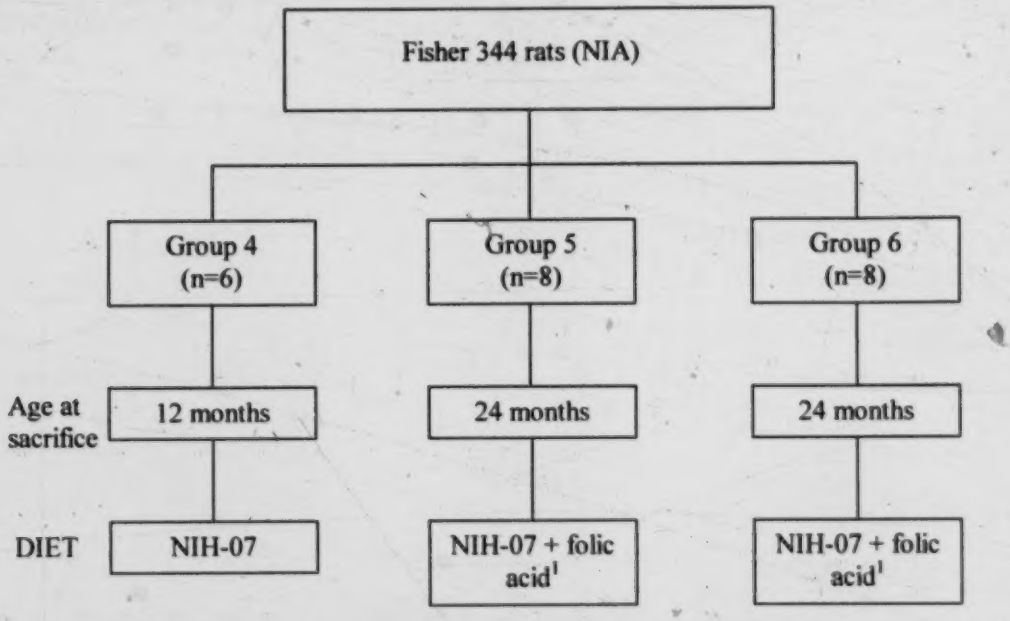
Vitamin /mineral	Amount per kg diet[2]
Thiamin	17.23 mg
Riboflavin	8.19 mg
Niacin	87.43 mg
Pyridoxine	10.83 mg
Vitamin B_{12}	77.77 μg
Folic acid	3.57 mg
Pantothenic acid	35.81 mg
Biotin	0.37 mg
Choline	2.19 mg
Vitamin A	9920 IU
Vitamin D_3	5070 IU
Menadione	9.34 mg
Cobalt	0.56 mg
Copper	13.67 mg
Iron	281.15 mg
Manganese	103.64 mg
Zinc	68.58 mg
Iodine	1.89 mg
Selenium	0.18 mg

[1]Supplied by Zeigler Bros., Inc. Gardeners. PA.

[2]Guaranteed analysis of diet, following the addition of vitamin and mineral premixes, provided by supplier for lot number used

Experiment 2: The male Fisher 344 rats (n = 22) used in the second study were obtained from the National Institute of Aging colonies (Harlan Spraque Dawley Inc. contractor, Indianapolis, IN) at 11 and 23 months of age. During a three-week acclimatization period all rats had free access to water and the NIH-07 diet described in Table 3. Following the acclimatization period the 11 month-old rats continued to receive the NIH-07 diet (n = 6), while the 23-month-old rats were randomly assigned to either the NIH-07 diet (n = 7) or to the NIH-07 diet supplemented with folic acid (Sigma Chemical, St. Louis, MO) to a final concentration of 35.7 g folic acid per kg diet (Figure 6). The folic acid content of this diet was 10 times higher than folic acid content of the basal NIH-07 diet. All animals had free access to food and water throughout the duration of the experiment. Food intake and body weight were recorded on alternate days. After one month of dietary treatment, rats were fasted overnight then anesthetized (3-5% halothane, 1.5-3.0 mL/min). Blood and liver samples were collected and stored as described for experiment 1. Both experiments were reviewed and approved by the Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee and conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Figure 6: Protocol of Experiment 2



[1]folic acid added to diet for a final folic acid concentration of 35.7 mg folic acid per kg

Materials and Methods

Hematology: A Coulter S+4 Hematology Analyzer (Fullerton, CA) was employed to determine packed cell volume (PCV), hemoglobin concentration, total red blood cell count (RBC), mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and total white blood cell counts (WBC). The differential white blood cell counts were determined manually on a smear of blood.

Clinical Chemistry: Plasma alanine aminotransferase (ALT), alkaline phosphatases (ALP), total bilirubin (TBIL), blood urea nitrogen (BUN), creatinine, glucose, albumin, globulin, sodium, potassium, carbon dioxide, calcium, phosphorus, and anion gap were measured. These tests were run on a Kodak 700XR Clinical Analyzer (Toronto, ON).

Vitamin Analysis

B_{12} and Folic Acid: Previously frozen plasma samples were thawed, only once, at room temperature. One hundred µL of plasma was required for each sample and sll samples were measured in triplicate. The commercially available Solid Phase No Boil Dualcount® kit (Diagnostic Products Corporation, Los Angeles, CA) was used for the determination of plasma vitamin B_{12} and folate concentrations. This competitive binding radioassay is designed for the simultaneous determination of the concentrations of plasma vitamin B_{12} and folic acid. The first step of the assay involved the alkaline (pH > 12) denaturation of endogenous plasma proteins in the presence of dithiothreitol and potassium cyanide. This step released the vitamin B_{12} and folic acid in the samples from their carrier proteins and inactivated any anti-intrinsic factor antibodies or vitamin B_{12} transport proteins present in the sample (Rothenberg et al., 1984). The addition of cyanide enhances the liberation of vitamin B_{12} from its binding proteins and converts hydroxycobalamin to the more stable cyanocobalamin thus protecting the newly released vitamin B_{12} (Rothenberg et al., 1984). Known quantities of ^{57}Co vitamin B_{12} and ^{125}I folic acid were added to the samples, then purified hog intrinsic factor and folate binding protein were added to act as binders for vitamin B_{12} and folic acid, respectively. The unlabeled vitamin B_{12} and folate compete with the labeled species for the available vitamin-specific binding sites. Thus, as the concentration of unlabeled endogenous vitamin increases, the amount of bound, radiolabled vitamin decreases. Vitamin B_{12} analogs do not interfere with this kit, as the binder employed is

free of R protein, which can bind with both cobalamin and cobalamin analogs (Kolhouse et al., 1978). Additionally, the reaction takes place at a pH where intrinsic factor is fully active and the folate binder has equal affinity for the predominant form of folate in the circulation (MTHF) and the form of folate used to create the calibration curves (polyglutamyl folic acid) (Sourial and Waters, 1992). After an adequate incubation period the bound vitamin fraction was isolated. As the binder provided is immobilized on microcrystalline cellulose this was easily accomplished by centrifugation (2000xg, 20 minutes) and aspiration of the supernatant. The remaining precipitate was counted on a dual-channel gamma counter (Packard 500C AutoGamma Counter, Packard Instrument Co., Meriden CT, USA). Counts in the precipitate were transformed, by comparison with a calibration curve, into vitamin B_{12} and folic acid concentrations.

Vitamin B_6: Plasma pyridoxal-5'-phosphate (PLP) and 4-pyridoxic acid (PA) concentrations were simultaneously determined by high performance liquid chromatography (HPLC) using the highly sensitive method of Kimura et al. (Kimura et al., 1996). One hundred µL of plasma was required for each sample and all samples were measured in duplicate. The extraction of free and protein-bound vitamers from plasma was achieved by the addition of perchloric acid (5% w/v) followed by vigorous vortexing. The precipitated protein was pelleted by centrifugation at 25 000 x g for 15 min and the resulting supernatant was reserved for HPLC analysis. A reverse-phase column and a mobile phase of 0.1 mol/L potassium dihydrogen phosphate (containing 0.1 mol/L sodium perchlorate and 0.5 g/L sodium bisulfite adjusted to pH 3) at a flow rate of 1.0 mL/min was used to separate the vitamers. The addition of the derivitizing reagent (sodium bisulfite) to the mobile phase results in a fluorescence intensity of PLP, 30 times greater than that obtained by post-column derivitization and minimizes the number of reagents required for the assay (Kimura et al., 1996). Retention time of PA was 3.33 minutes and PLP eluted at 22.68 minutes. A fluorometric detector was used to measure the fluorescence intensity of PLP and PA (excitation wavelength 300 nm, emission wavelength 400 nm). All assay runs were calibrated with calibrators positioned at random along with the test samples and results demonstrated the consistent reliability of the data throughout the assay.

Plasma Amino Acid Analysis

Homocysteine and Cysteine: Blood samples collected for homocysteine analysis were immediately placed on ice and protected from light. Within 2 hours of sampling the plasma was separated and stored at −70°C until subsequent analysis, at which point they were thawed, only once, at room temperature. Handling samples in this manner prevented the production and release of homocysteine into the plasma by red blood cells, which can increase plasma homocysteine by as much as 10% per hour (Ueland et al., 1993). Three hundred µL of plasma was required for each sample and all samples were measured in duplicate. The concentrations of homocysteine and cysteine in plasma were determined using a slight modification of the HPLC method of Araki and Sako (1987). The most widely used and accepted measurement of plasma homocysteine is that of total plasma homocysteine, which includes free plasma homocysteine, homocystine, protein-bound homocysteine and the disulfide cysteine-homocysteine. Samples were pretreated by incubation with the reducing agent tri-η-butylphosphine to convert the disulphides and protein-bound thiols to free homocysteine and other thiols. Plasma proteins were then precipitated with perchloric acid; thiol groups were derivatized with the thiol-specific, highly fluorogenic reagent ammonium-7-fluorobenzo-2-oxa1,3-dizole-4-sulfonate (SBDF) and the resulting mixture was centrifuged. SBDF labeled thiol derivatives remaining in the supernatant were separated by reverse-phase HPLC and quantified by fluorometric detection (excitation wavelength 385 nm, emission wavelength 515 nm). All assay runs were calibrated with calibrators positioned randomly among the test samples, and the results demonstrated consistent reliability of the data throughout the assay.

Methionine: Plasma methionine concentration was determined along with other plasma amino acids (aspartic acid, glutamic acid, asparagine, serine, glutamine, histidine, glycine, threonine, citruline, arginine, taurine, alanine, tyrosine, tryptophan, valine, phenylalanine, isoleucine, lysine) by the HPLC method of Jones and Gilligan (1983) with modifications described by Sedgwick et al. (1991). The precipitation of plasma proteins was achieved by the addition of trichloroacetic acid (5% w/v) followed by vigorous vortexing. The precipitated protein was pelleted by centrifugation at 25 000 x g for 15 min and the resulting supernatant was reserved for HPLC analysis. Two amino acid standard solutions were used: the first was prepared by diluting cysteic acid and methionine sulfone in deionized water, the second was prepared by adding asparagine, glutamine, citrulline, taurine and tryptophan to a standard protein hydrolysate solution (Sigma Chemical Co., St. Louis, MO); the final concentration of each amino acid in the standard

solutions was 100 μM. Precipitated plasma samples and standards were mixed 1:1 with the derivitizing reagent prior to injection on to a 4.6 X 150 mm reverse-phase Supelcosil 3 micron LC-18 column (Supelco; Oakville, ON) equipped with a 4.6 X 50 mm guard column containing Supelco LC-18 reverse-phase packing (20-40 μm). The derivitizing reagent contained 0.25 g o-phthaldialdehyde (OPA), 56 mL sodium borate buffer (pH 9.5), 0.25 mL mercaptoethanol and 2 mL Brij 35. In the presence of mercaptan, OPA reacts rapidly with primary amino acids to form intensely fluorescent derivatives (Jones and Gilligan, 1983). The mobile phase was comprised of two solvents with a total flow rate of 1.1 mL/min. For the determination of methionine levels solvent A consisted of 0.1M sodium acetate (pH 6.8): methanol: tetrahydrofuran in a ratio of 940:55:5 and solvent B was methanol. For the determination of the other plasma amino acids levels solvent A consisted of 0.1M sodium acetate (pH 7.2): methanol: tetrahydrofuran in a ratio of 905:90:5 and solvent B was methanol. The gradient used was 0% B to 19% B in 0.1 min, 19% B for 14.9 min, 19% B to 32% B in 10 min, 32% B to 42% B in 0.1 min, 42% B to 50% B in 7.9 min, 50% B to 70% B in 7 min 70% B to 100 % B in 2 min, 100% B for 2 min, 100% b to 0% B in 1 min, with a total analysis time of 52 min. A fluorometric detector was used to measure the fluorescence intensity of the amino acids (excitation wavelength 340 nm, emission wavelength 450 nm). Chromatographic peaks were recorded and integrated using a Shimadzu Class-VP Chromatography Data System (Shimadzu Scientific Instrument Inc., Columbus, MD, USA).

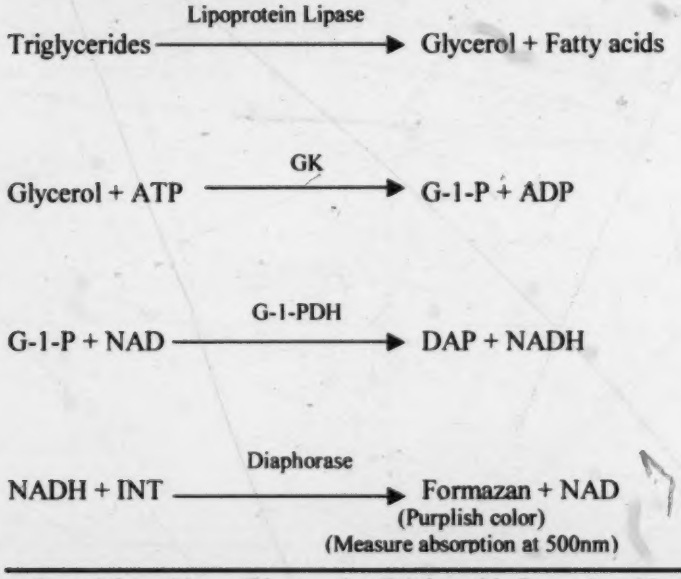
Plasma and Liver Lipids

Extraction of Liver Lipids: Lipids were extracted from approximately 1g of liver tissue using the method of Folch *et al* (1957). Liver samples were homogenized, for 2 minutes, with 5 mL sterile saline and 20 mL cholorform:methanol (2:1). The homogenate was then vortexed, centrifuged (100xg, 10 minutes) and left overnight at 4°C to achieve good phase separation. The upper methanol and water layer was aspirated off and discarded. The lower layer, containing the lipids, was dried under liquid nitrogen then reconstituted with isopropanol. The extraction process was repeated on the remaining homogenate in an effort to maximize lipid recovery.

Determination of Plasma and Liver Triglyceride: Plasma and liver triglyceride content was determined using Sigma Diagnostics kit #336 (St. Louis, MO). Ten microliters μL of plasma or liver lipid extract were required for each sample and all samples were measured in triplicate. This enzymatic procedure (Figure 7) is a modification of the method of Bucola and David (1973). In short, lipoprotein lipase hydrolyzes the triglycerides in the sample to glycerol and free fatty

acids. Then glycerol kinase (GK) catalyzes the ATP dependent phosphorylation of glycerol to glycerol-1-phosphate (G-1-P). The next reaction utilizes glycerol-1-phosphate dehydrogenase (G1P-DH) to catabolize the concomitant oxidation of G-1-P to dihydroxyacteone phosphate (DAP) and the reduction of nicotinamide adenine dinucleotide (NAD) to NADH. Diaphorase then oxidizes the NADH, while simultaneously reducing 2[-p-iodophenyl]-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT) to the highly colored product, formazan (INTH, max absorbance 500 nm). The concentration of triglyceride in the sample is directly proportional to the intensity of the color produced.

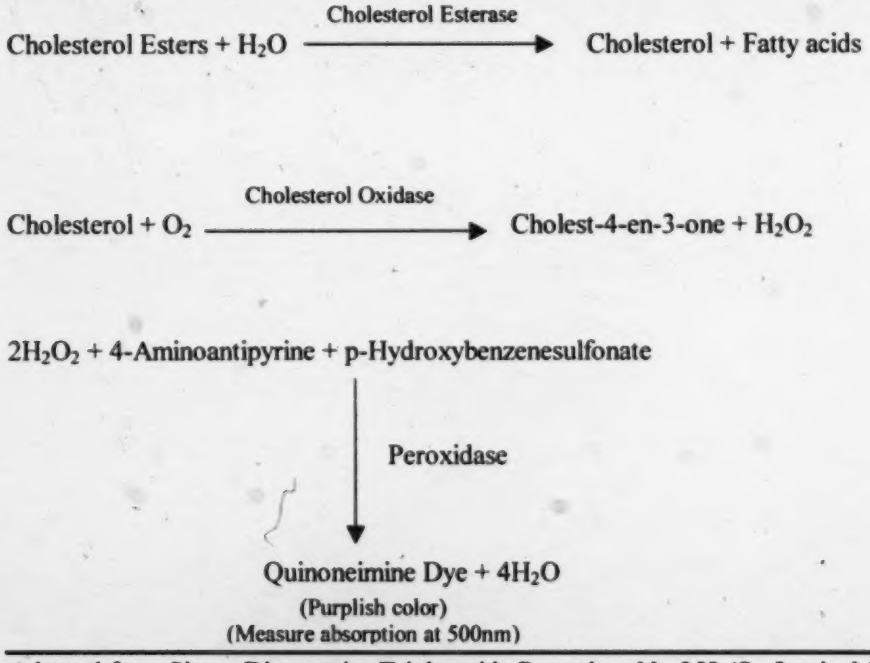
Figure 7: Enzymatic reactions involved in the determination of plasma and liver triglyceride concentrations



Adapted from Sigma Diagnostics Triglyceride Procedure No.336 (St. Louis, MO)

Determination of Plasma and Liver Total Cholesterol: Plasma and liver total cholesterol content was determined using Sigma Diagnostics kit #352 (St. Louis, MO). Ten microliters μL of plasma or liver lipid extract were required for each sample and all samples were measured in triplicate. This enzymatic procedure (Figure 8) is a modification of the method of Allain *et al.* (1974). Briefly, cholesterol esterase hydrolyzes the cholesterol esters in the sample to free cholesterol and fatty acids. Then cholesterol oxidase catalyzes oxidation of free cholesterol to cholest-4-en-3-one and hydrogen peroxide (H_2O_2). The hydrogen peroxide produced couples with the chromogen, 4-aminoantipyrine and p-hydroxybenzenesulfonate in a reaction catalyzed by peroxidase. This yields a quinoneimine dye with an absorbance maximum of 500 nm. The concentration of cholesterol in the sample is directly proportional to the intensity of the color produced.

Figure 8: Enzymatic reactions involved in the determination of plasma and liver cholesterol concentrations



Adapted from Sigma Diagnostics Triglyceride Procedure No.352 (St. Louis, MO)

Statistical Analysis

All statistical analysis was performed using the Statistical Analysis System (SAS) software package (version 7.0, SAS Institute, Cary, NC). Data are expressed as means ± standard error of the mean (SEM) and the level of significance was set at $p \leq 0.05$. The amino acids, B-vitamins, plasma and liver lipids, hematology, and blood chemistry data were analyzed for the effects of age or diet using proc GLM and one-way analysis of variance (ANOVA). Differences were identified using least square means/pdiff. Pearson correlation coefficients (R) were determined to identify significant correlations between age, plasma B-vitamin levels, B-vitamin intake, plasma and liver lipid concentrations and plasma sulfur amino acid concentrations.

CHAPTER 4: RESULTS

During the acclimatization period two of the 24 month-old animals died. While the experiment was in progress an 18 month-old rat was euthanized following the appearance of blood on the urine. One 24 month-old animal was excluded from the study when post-mortem examination revealed tumors throughout the abdomen. Two other 24 month-old animals were excluded from the study when clinical chemistry results revealed extremely elevated blood levels creatinine and serum urea nitrogen. No data from these animals was included in the statistical analysis.

Experiment #1:

Fisher 344 rats of three age groups, 12, 18 and 24 months, were included in this experiment. At the time these animals were transferred to our laboratory, the body weights were approximately 9% higher ($p < 0.05$) in both 18 and 24 month-old than 12 month-old rats (Table 5). After 3 weeks of acclimatization, the body weight gains of these rats were restricted with increasing age, while their daily food intakes remained similar in all three age groups. Like food intake, the age did not have any appreciable effect on the liver weight in relation to the body weight.

Table 5. Age-associated changes in food intake and body weights of Fisher 344 rats

	Age (months)		
	12 (n = 6)	18 (n = 5)	24 (n = 4)
Initial body weight[1] (g)	428 ± 11^{b}	468 ± 11^{a}	465 ± 12^{a}
Body weight change[2] (g)	21.05 ± 3.34^{a}	9.73 ± 3.34^{b}	-2.10 ± 3.66^{c}
Liver weight (% final BW)[3]	3.01 ± 0.13	3.14 ± 0.14	3.31 ± 0.16
Food intake (g/d)	15.7 ± 0.6	14.9 ± 0.6	14.2 ± 0.6

Values presented are those of lsmeans of each age group, ± SEM. Values that do not share the same superscript letter within a row are significantly different ($p<0.05$).

1 Body weight at beginning of 3-week acclimatization period

2 Body weight change = body weight at time of sacrifice - body weight at beginning of acclimatization period

3 BW = body weight

The influence of age on some hematological indices is presented in Table 6. The parameters reflecting hemoglobin status and the counts of both red and white blood cells fell within the normal ranges, irrespective of age differences. Of these indices, it was only the mean corpuscular hemoglobin concentration that was significantly higher in the 18 or 24 month-old rats compared to their 12 month-old counterparts. However, even these values were still within the normal ranges.

Table 6: Age associated changes in hematological parameters of Fisher 344 rats

		Age (months)		
	Normal range[1]	12 (n = 6)	18 (n = 5)	24 (n = 4)
Hemoglobin (g/L)	140-152	149 ± 3	149 ± 3	149 ± 3
Hematocrit (L/L)	0.35-0.51	0.45 ± 0.01	0.44 ± 0.01	0.43 ± 0.01
Red blood cells (tera/L)	6.26-8.74	8.66 ± 0.22	8.42 ± 0.23	8.19 ± 0.25
MCV[2] (fL)	53-58	52.4 ± 0.6	52.3 ± 0.6	53.0 ± 0.7
MCH[3] (pg)	18-23	17.3 ± 0. 3	17.8 ± 0.3	18.2 ± 0.3
MCHC[4] (g/L)	320-409	330 ± 3[b]	339 ± 3[a]	344 ± 3[a]
White blood cells (/mm^3)	3400-6000	5540 ± 845	4250 ± 772	5425 ± 945
Lymphocytes (/mm^3)	2444-3948	3584 ± 643	2716 ± 587	3463 ± 719
Neutrophils (/mm^3)	1360-3600	1813 ± 410	1464 ± 374	1739 ± 459
Monocytes (/mm^3)	0-240	89.7 ± 39.7	44.5 ± 58.3	126.6 ± 41.2
Eosinophils (/mm^3)	0-120	87.7 ± 23.9	65.4 ± 19.6	106.2 ± 23.9

Values presented are those of lsmeans of each age group, ± SEM. Values that do not share the same superscript letter within a row are significantly different ($p<0.05$).

1 Normal ranges for Fisher 344 rats obtained from Charles River Laboratories (1984)

2 Mean corpuscular volume

3 Mean corpuscular hemoglobin

4 Mean corpuscular hemoglobin concentration

Similar to the hematological indices, the serum parameters reflecting renal function fell within the normal ranges (Table 7). Of all the indices tested the serum levels of creatinine, calcium and chloride were elevated ($p < 0.05$) in 24 month-old rats compared to the younger rats.

Table 7: The effects of age on serum indicators of renal function in Fisher 344 rats

		Age (months)		
	Normal range[1]	12 (n=6)	18 (n=5)	24 (n=4)
BUN (mmol/L)	3.5-8.0	5.16 ± 0.53	5.05 ± 0.49	5.60 ± 0.60
Creatinine (μmol/L)	26-80	29.4 ± 3.4^{b}	31.78 ± 3.1^{b}	49.08 ± 3.8^{a}
Calcium (mmol/L)	2.16-2.95	2.46 ± 0.03^{b}	2.53 ± 0.02^{ab}	2.58 ± 0.03^{a}
Potassium (mmol/L)	4.5-9.5	4.94 ± 0.21	5.00 ± 0.20	4.52 ± 0.24
Sodium (mmol/L)	130-151	143.0 ± 0.5	143.0 ± 0.4	141.7 ± 0.5
Chloride (mmol/L)	96-112	102.6 ± 0.3^{b}	102.7 ± 0.3^{b}	104.8 ± 0.4^{a}

Values presented are those of lsmeans of each age group, ± SEM. Values that do not share the same superscript letter within a row are significantly different ($p<0.05$).

[1] Normal ranges for Fisher 344 rats obtained from Charles River Laboratories (1984)

In order to determine if aging has any effect on the hepatic function, serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin were determined in 12, 18 and 24 month-old Fisher 344 rats. These values were all within the normal ranges (Table 8), however, the values for ALT were lower ($p <0.05$) in 24 month-old than in either 12 or 18 month-old animals (Table 8).

Table 8: The effects of age on serum indicators of hepatic function in Fisher 344 rats

		Age (months)		
	Normal range[1]	12 (n=6)	18 (n=5)	24 (n=4)
ALT (IU/L)	10-240	77.8 ± 8.1^{a}	59.1 ± 7.4^{ab}	49.7 ± 9.1^{b}
ALP (IU/L)	98-224	191 ± 11	171 ± 10	194 ± 12
Total bilirubin (μmol/L)	6-28	16.8 ± 1.9	16.8 ± 1.8	15.0 ± 2.2

Values presented are those of lsmeans of each age group, ± SEM. Values that do not share the same superscript letter within a row are significantly different ($p<0.05$).

[1] Normal ranges for Fisher 344 rats obtained from Charles River Laboratories (1984)

The hepatic concentrations of total cholesterol and triglycerides did not differ significantly between 12 and 18 or 24 month-old rats (Table 9). However, the serum total cholesterol concentration was elevated in an age-related fashion, although the difference was statistically significant ($p < 0.05$) only between 12 and 18 vs. 24 month-old rats. Correlation analysis revealed a direct relationship between total plasma cholesterol concentration and age ($r=0.77$, $p=0.0005$). Plasma triglyceride levels, however, were not significantly different between rats of different ages (Table 9).

Table 9: The effects of age on plasma and hepatic lipid concentrations in Fisher 344 rats

	Age (months)		
	12 (n=6)	18 (n=5)	24 (n=5)
Liver cholesterol (mg/g liver)	3.52 ± 0.20	3.60 ± 0.22	3.27 ± 0.22
Liver triglyceride (mg/g liver)	24.7 ± 2.6	25.8 ± 3.2	21.6 ± 3.2
Plasma cholesterol (mg/dL)	55.7 ± 10.1^{b}	87.6 ± 12.3^{b}	127.1 ± 12.3^{a}
Plasma triglyceride (mg/dL)	164 ± 27	176 ± 33	202 ± 33

Values presented are those of lsmeans of each age group, ± SEM. Values that do not share the same superscript letter within a row are significantly different ($p<0.05$).

The effect of aging on plasma homocysteine and cysteine levels is presented in Figures 9 and 10, respectively. No significant differences in the amino acid concentrations were found between the 12 and 18 month-old rats. Plasma homocysteine levels, however, were significantly ($p < 0.05$) higher in the 24 month-old rats than in the younger rats (Figure 9). Plasma levels of cysteine (Figure 10) and the molar ratio of homocysteine to cysteine (Figufe 11) did not significantly differ between rats of different age groups. However, it should be pointed out that, although not statistically significant, the latter was increased in response to aging, apparently due to the increase in homocysteine with age.

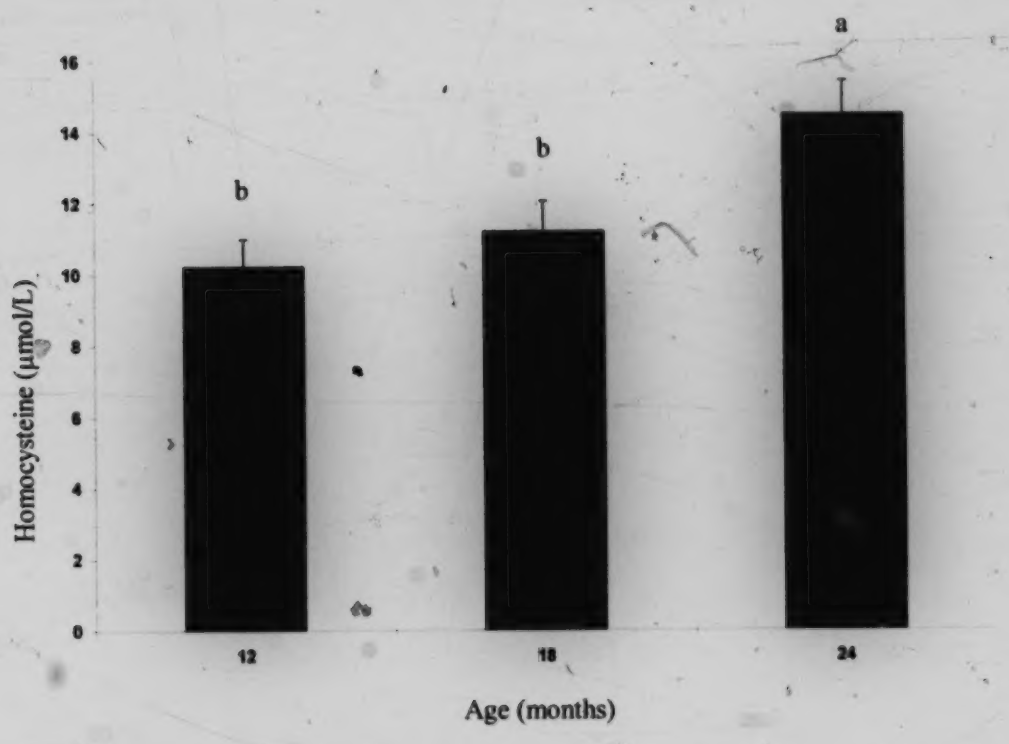


Figure 9: The effect of age on plasma homocysteine concentrations in Fisher 344 rats

Values presented are those of lsmeans of each age group, ± SEM (n = 6 for 12 month-old rats, n= 5 for 18 month-old rats, n = 5 for 24 month-old rats). Bars that do not share the same superscripts are significantly different ($p<0.05$).

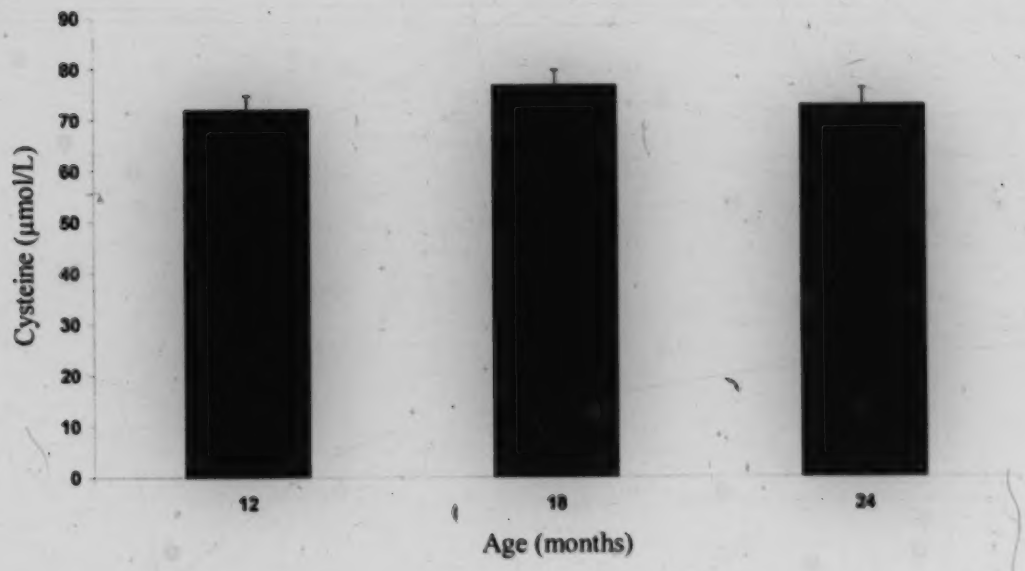


Figure 10: The effect of age on plasma cysteine concentrations in Fisher 344 rats

Values presented are those of lsmeans of each age group, ± SEM (n = 6 for 12 month-old rats, n= 5 for 18 month-old rats, n = 5 for 24 month-old rats). Plasma cysteine concentration did not differ significantly between groups ($p>0.05$).

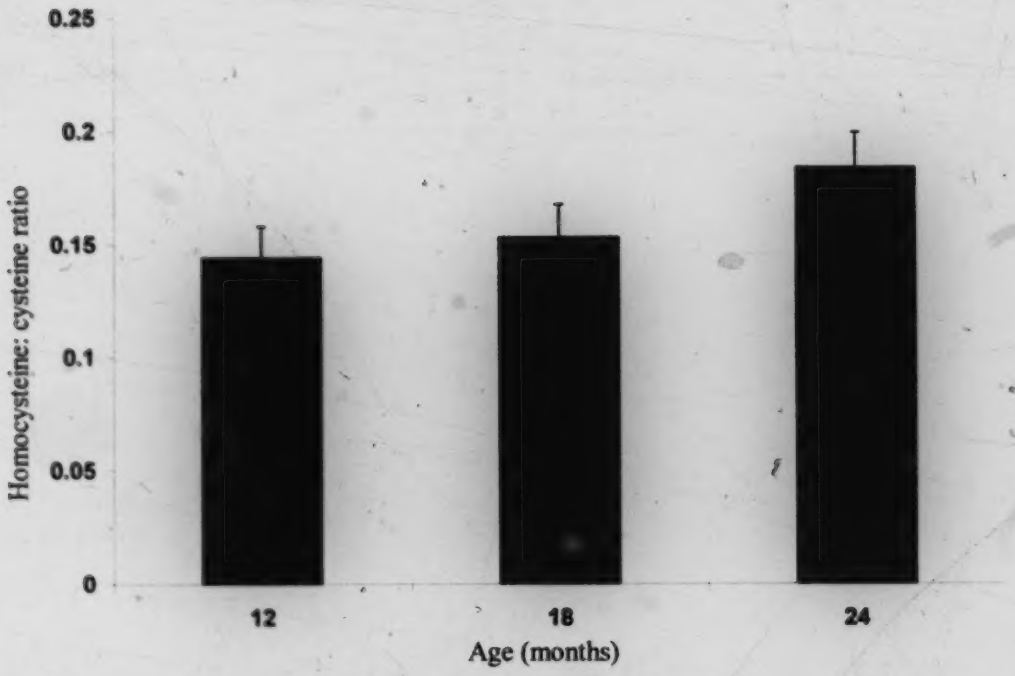


Figure 11: The effect of age on ratio of plasma homocysteine to cysteine in Fisher 344 rats

Values presented are those of lsmeans of each age group, ± SEM (n = 6 for 12 month-old rats, n= 5 for 18 month-old rats, n = 5 for 24 month-old rats). Molar ratio of plasma homocysteine to cysteine did not differ significantly between groups ($p>0.05$).

The modifying effects of aging on the biochemical evidence of B-vitamin status, with particular reference to the vitamins involved in homocysteine metabolism, which includes folate, vitamin B_{12} and vitamin B_6, are presented in Table 10. Plasma folate concentrations were significantly higher in the 12 month-old than in the 18 or 24 month-old rats. In contrast to folate, there was a significantly elevated level of plasma vitamin B_{12} in the 24 month-old animals compared to the 12 or 18 month-old rats. Plasma concentrations of PLP, the active form of vitamin B_6, were increased at 18 months, followed by a decrease at 24 months. The plasma level of its metabolic end product, pyridoxic acid, however, remained unaffected by the age difference.

Table 10: The effect of age on plasma B-vitamin concentrations in Fisher 344 rats

	Age (months)		
	12 (n=6)	18 (n=5)	24 (n=5)
Folate (nmol/L)	230 ± 8^a	197 ± 10^b	192 ± 10^b
Vitamin B_{12} (pmol/L)	709 ± 35^b	751 ± 43^b	931 ± 43^a
Pyridoxal phosphate (nmol/L)	223 ± 16^b	293 ± 19^a	195 ± 19^b
Pyridoxic acid (nmol/L)	10.9 ± 0.6	12.2 ± 0.7	10.7 ± 0.7

Values presented are those of lsmeans of each age group, ± SEM. Values that do not share the same superscript letter within a row are significantly different ($p<0.05$).

Homocysteine concentrations were correlated directly with age, serum creatinine and plasma cholesterol levels but not with plasma B-vitamin concentrations (Table 11). Plasma vitamin B_{12} levels were positively correlated, while folate concentrations were inversely correlated with age. Multiple regression analysis revealed age to be the only significant predictor of homocysteine levels (Figure 12). Plasma total cholesterol concentrations were correlated with plasma vitamin B_{12} concentration ($r = 0.81$, $p = 0.0001$), age ($r = 0.77$. $p = 0.0005$), and homocysteine ($r = 0.60$, $p = 0.0136$).

Table 11: Correlations of selected plasma and serum metabolites with age and plasma homocysteine concentrations in Fisher 344 rats

		Age	Homocysteine
Homocysteine	r[1]	0.67	1.00
	p[2]	0.0046	--
Creatinine	r	0.69	0.56
	p	0.0043	0.0303
Folate	r	-0.60	-0.39
	p	0.0137	0.1398
Vitamin B_{12}	r	0.70	0.52
	p	0.0025	0.0384
PLP	r	-0.13	-0.09
	p	0.6254	0.7508
Pyridoxic acid	r	0.02	0.23
	p	0.9541	0.3773

[1] r = Pearson correlation coefficient

[2] p =. Significance level

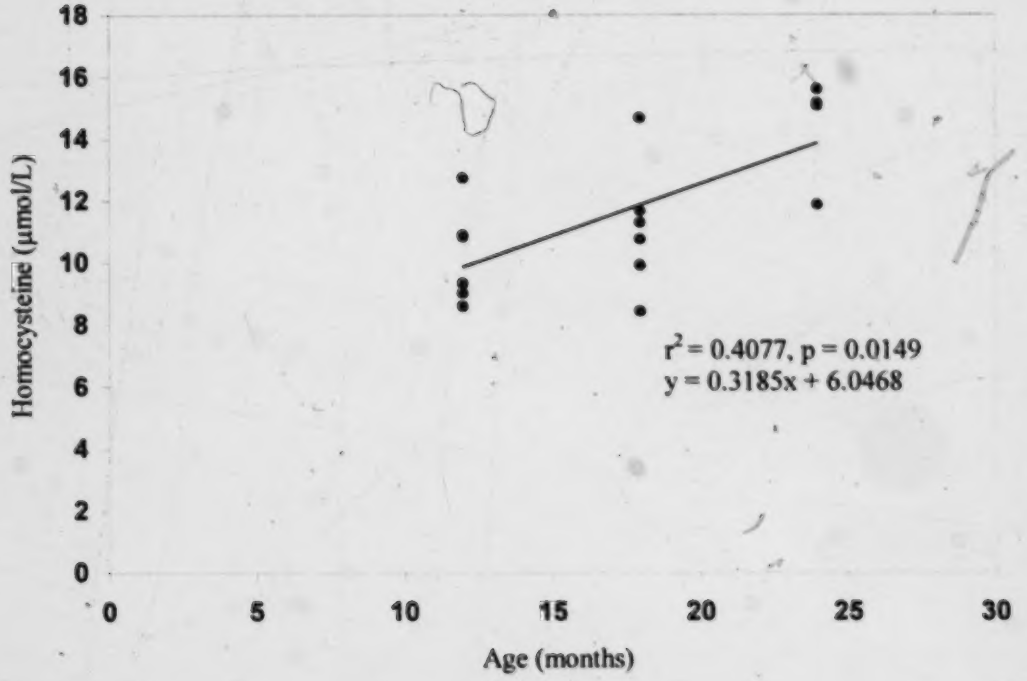


Figure 12: Linear regression analysis of plasma homocysteine concentration vs. age in 12, 18, and 24 month-old Fisher 344 rats

(y = plasma homocysteine concentration, x = age)

Experiment #2:

Fisher 344 rats of two age groups, 12 and 24 months, were included in this experiment. The older animals were maintained for four weeks with or without dietary folic acid supplementation. At the beginning of the experimental period the body weights were similar in all age groups (Table 12). During the following four weeks the magnitude of weight gain restriction was more pronounced in 24 month-old than in 12 month-old rats. Dietary folic acid supplementations to the older rats did not reverse this age-associated weight gain restriction. In parallel with the changes in body weight gain the daily food intake was reduced in older rats, irrespective of folic acid supplementation (Table 12). However, when the daily food intake was expressed as a function of body weight no significant difference in food intake was found between the two age groups. Age did not have any appreciable affect on the liver weight, especially in relation to the body weight (Table 12).

Table 12. Effect of folic acid supplementation on food intake and body weight of Fisher 344 rats

	Age (months)		
	12 (n = 6)	24 (n = 6)	24 (n = 6)
Diet	NIH-07	NIH-07	NIH-07 + Folic acid[1]
Initial body weight[2] (g)	425 ± 12	459 ± 12	457 ± 12
Body weight change[3] (g)	11.1 ± 3.4[a]	-3.9 ± 3.4[b]	-3.0 ± 3.4[b]
Liver weight (% final BW)[4]	2.96 ± 0.12	2.90 ± 0.12	2.70 ± 0.12
Food intake (g/d)	20.8 ± 0.5[a]	18.4 ± 0.5[b]	18.3 ± 0.5[b]
Food intake per BW (mg/g)	45.2 ± 2.0	41.0 ± 2.0	41.1 ± 2.0

Values presented are those of lsmeans of each age/diet group, ± SEM. Values that do not share the same superscript letter within a row are significantly different ($p<0.05$).

1 Folic acid added to NIH-07 diet for a final folic acid content of 35.7mg/Kg diet

2 Body weight at beginning of experiment

3 Body weight change = body weight at time of sacrifice - body weight at beginning of experiment

4 BW = body weight

The hematological and serological indices, described in the first experiment (tables 6-8), remained unchanged ($p>0.05$) by supplemental intake of folic acid for four weeks. Thus, the age-associated increase in MCHC or the serum levels of creatinine and calcium were unchanged in the presence of dietary folate supplementation.

In parallel with the first experiment (Table 9), the hepatic concentrations of total cholesterol and triglycerides as well as the plasma levels of triglycerides were similar in 12 and 24 month-old rats (Table 13). Furthermore, as demonstrated in the first experiment, plasma total cholesterol concentration was significantly higher in the older rats than in the younger rats (Table 13). Supplemental intake of folic acid for four weeks failed to reverse the age-associated changes in plasma cholesterol levels.

Table 13: The effect of folic acid supplementation on plasma and hepatic lipid concentrations in Fisher 344 rats

	Age (months)		
	12 (n=6)	24 (n=6)	24 (n=6)
Diet	NIH-07	NIH-07	NIH-07 + Folic acid[1]
Liver cholesterol (mg/g liver)	2.59 ± 0.27	2.80 ± 0.26	2.95 ± 0.26
Liver triglyceride (mg/g liver)	16.9 ± 2.2	12.5 ± 2.2	13.6 ± 2.2
Plasma cholesterol (mg/dL)	77.5 ± 10.7[b]	145.3 ± 10.7[a]	161.50 ± 10.7[a]
Plasma triglyceride (mg/dL)	129 ± 18	177 ± 18	186 ± 18

Values presented are those of lsmeans of each age/diet group, ± SEM. Values that do not share the same superscript letter within a row are significantly different ($p<0.05$).

[1] Folic acid added to NIH-07 diet for a final folic acid content of 35.7mg/Kg diet

As shown in Figure 13, the plasma homocysteine levels were significantly higher in 24 month-old than in 12 month-old rats. This difference, however, disappeared when the older animals were fed a diet containing folic acid (35.7 mg/Kg) for four weeks. Plasma levels of cysteine, conversely, remained similar, irrespective of age difference and folic acid intake (Figure 14). The molar ratio of homocysteine to cysteine did not differ neither between 12 and 24 month-old rats, nor the 24 month-old groups receiving a diet with or without folic acid supplementation. However, due to changes in homocysteine levels with age and folic acid supplementation, the molar ratio of homocysteine to cysteine followed a trend similar to that of homocysteine levels (Figure 15). In addition to homocysteine and cysteine, plasma methionine was determined in an effort to further explore the metabolic fate of homocysteine in these rats. Plasma methionine levels were similar between 12 and 24 month-old rats. Dietary supplemental intake of folic acid for four weeks had no influence on the methionine status in 24 month-old rats compared to their unsupplemented counterparts (Figure 16).

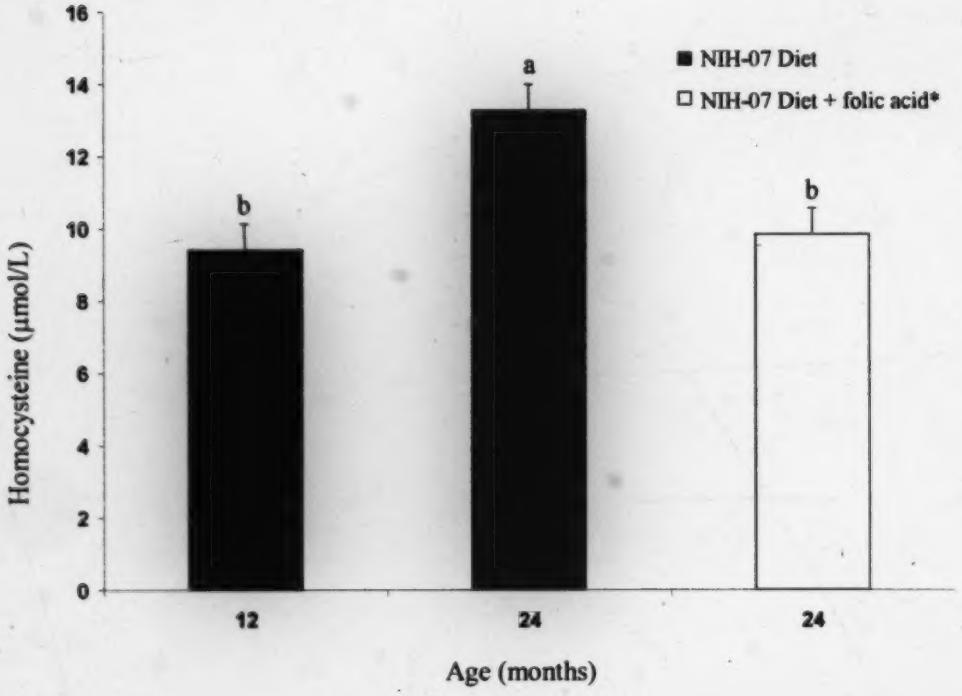


Figure 13: Effect of folic acid supplementation on age-associated changes in plasma homocysteine concentrations in Fisher 344 rats

Values presented are those of lsmeans of each age/diet group, ± SEM (n = 6 per group). Bars that do not share the same superscripts are significantly different ($p<0.05$).

*Folic acid added to NIH-07 diet for a final folic acid content of 35.7mg/Kg diet

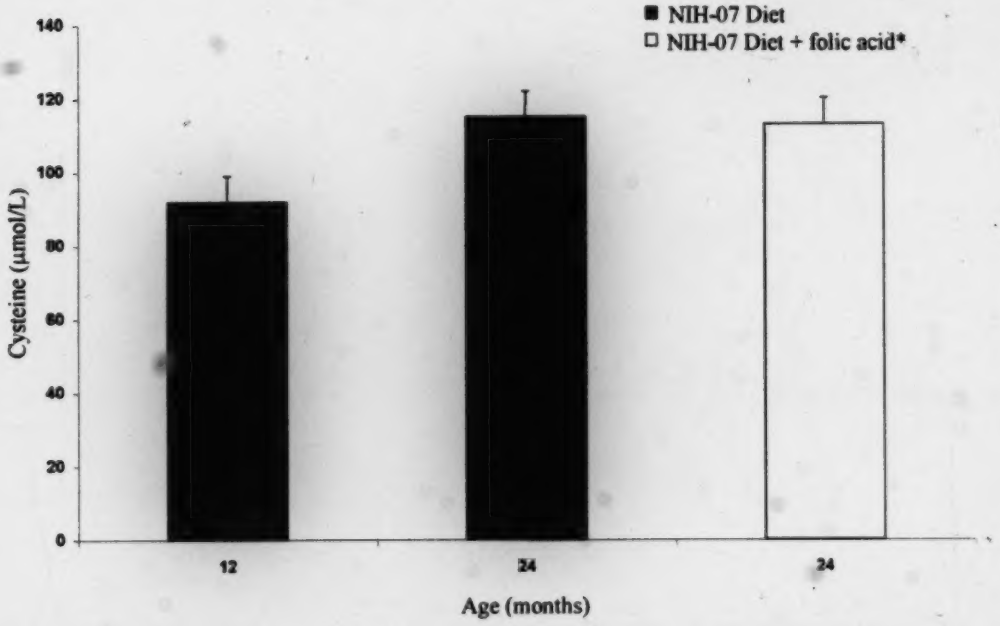


Figure 14: Effect of folic acid supplementation on age-associated changes in plasma cysteine concentrations in Fisher 344 rats

Values presented are those of lsmeans of each age/diet group, ± SEM (n = 6 per group). Plasma cysteine concentration did not differ significantly between groups ($p>0.05$).

*Folic acid added to NIH-07 diet for a final folic acid content of 35.7mg/Kg diet

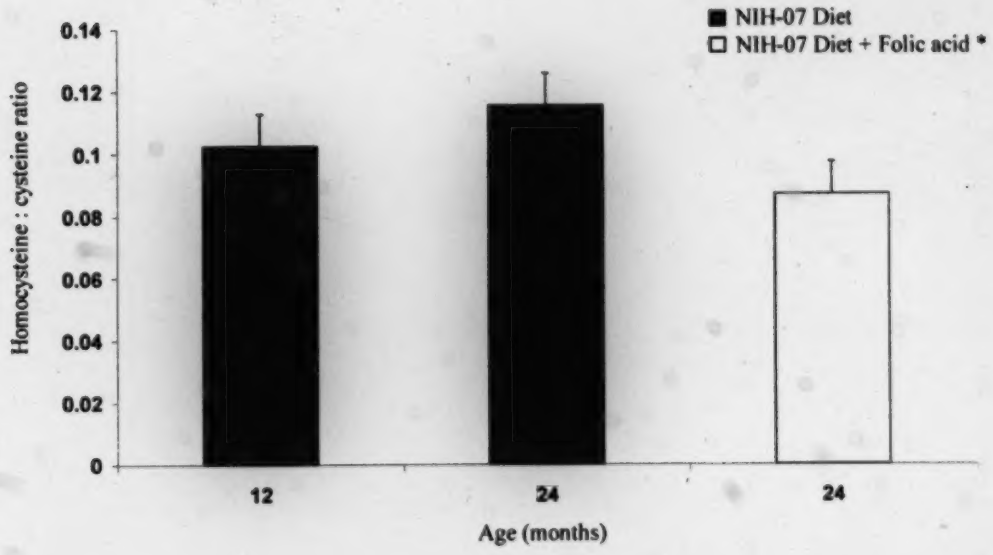


Figure 15: Effect of folic acid supplementation on age-associated changes in molar ratio of concentration of plasma homocysteine to cysteine in Fisher 344 rats

Values presented are those of lsmeans of each age/diet group, ± SEM (n = 6 per group). Molar ratio of plasma homocysteine to cysteine did not differ significantly between groups ($p>0.05$).

*Folic acid added to NIH-07 diet for a final folic acid content of 35.7mg/Kg diet

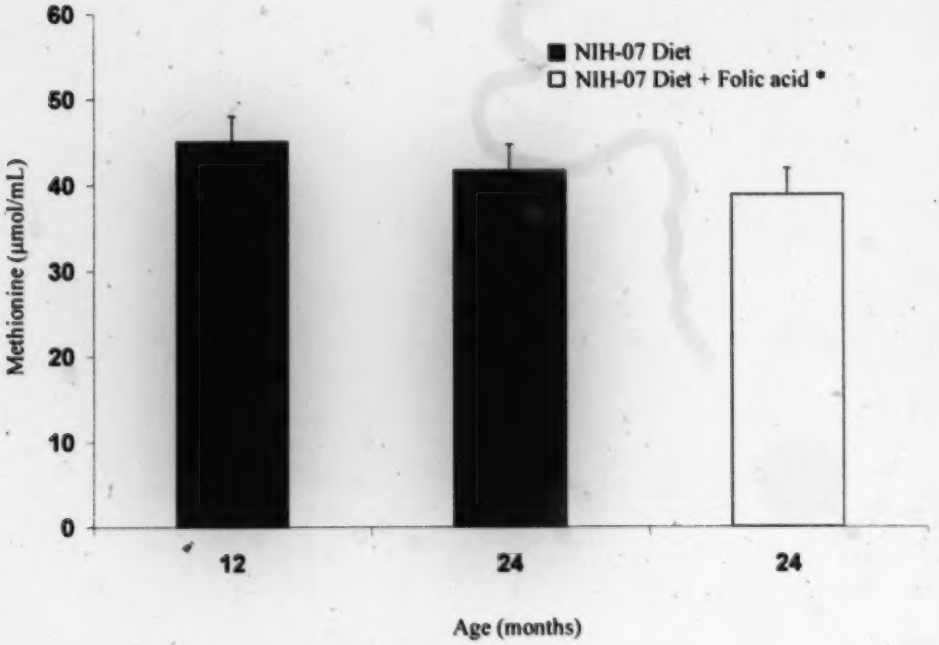


Figure 16: Effect of folic acid supplementation on age-associated changes in plasma methionine concentration in Fisher 344 rats

Values presented are those of lsmeans of each age/diet group, ± SEM (n = 6 per group). Plasma methionine concentration did not differ significantly between groups ($p>0.05$).

*Folic acid added to NIH-07 diet for a final folic acid content of 35.7mg/Kg diet

Supplemental intake of folic acid for four weeks resulted in an elevation of plasma folate concentrations in both 12 and 24 month-old (Table 14). The estimated folic acid intake of the 24 month-old rats was significantly correlated with plasma folate levels ($r = 0.80$, $p = 0.003$). The relationship between folic acid intake and plasma folate values was further assessed by linear regression (Figure 17).

There was a trend ($p = 0.060$) towards higher plasma vitamin B_{12} values in 24 month-old than in 12 month-old unsupplemented rats (Table 14). The levels in 24 month-old rats, following folic acid supplementation for four weeks, were higher than 12 month-old unsupplemented rats (Table 14). Neither PLP nor pyridoxic acid concentrations in plasma were affected by supplemental intake of folic acid.

Table 14: The effect of folic acid supplementation on plasma B-vitamin concentrations in Fisher 344 rats

	Age (months)		
	12 (n = 6)	24 (n = 6)	24 (n = 6)
Diet	NIH-07	NIH-07	NIH-07 + Folic acid[1]
Folate (nmol/L)	242 ± 20^{b}	190 ± 20^{b}	348 ± 20^{a}
Vitamin B_{12} (pmol/L)	861 ± 79^{b}	1094 ± 79^{ab}	1264 ± 79^{a}
Pyridoxal phosphate (nmol/L)	225 ± 15	252 ± 15	215 ± 15
Pyridoxic acid (nmol/L)	9.01 ± 1.06	6.78 ± 1.06	8.05 ± 1.06

Values presented are those of lsmeans of each age/diet group, ± SEM. Values that do not share the same superscript letter within a row are significantly different ($p<0.05$).

[1] Folic acid added to NIH-07 diet for a final folic acid content of 35.7mg/Kg diet

Plasma homocysteine concentrations within the 24 month-old rats were correlated inversely with both plasma folate concentrations ($r = -0.69$, $p = 0.0127$) and estimated folic acid intakes ($r = -0.72$, $p = 0.0083$). Neither plasma vitamin B_{12} and PLP nor creatinine levels were significantly correlated with homocysteine concentrations. Multiple regression analysis revealed folic acid intake to be the strongest predictor of plasma homocysteine, followed closely by plasma folate concentration ($r^2 = 0.5557$, $p = 0.0260$). Plasma total cholesterol and triglyceride concentrations were correlated with plasma vitamin B_{12} concentrations ($r = 0.58$, $p = 0.0.0489$ and $r = 0.73$, $p = 0.0068$, respectively). Cholesterol data from both experiments were pooled to further explore the relationships between plasma total cholesterol and homocysteine concentrations as well as age. There was a significant positive relationship between age and plasma cholesterol (Figure 18). The relationship remained unchanged when the animals that received folic acid supplementation for 4 weeks were included (Figure 19). There was also a significant positive relationship between plasma homocysteine and cholesterol in all age groups of rats (Figure 20). However, the addition of folic acid to the diet of the older rats resulted in the decline of this relationship to a non-significant level (Figure 21).

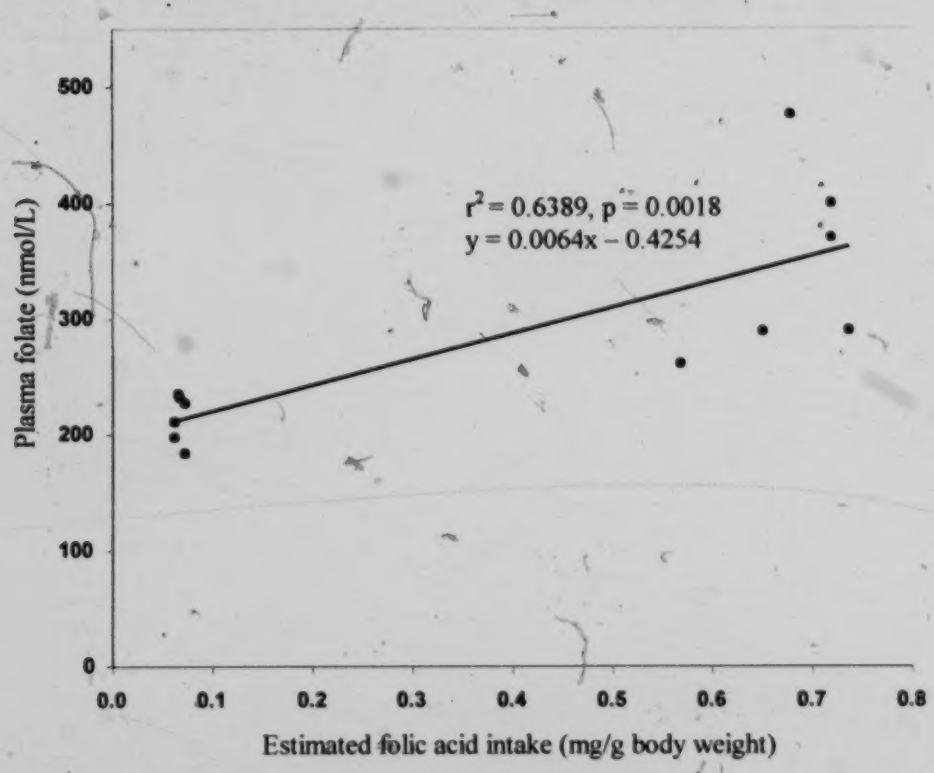


Figure 17: Linear regression analysis of estimated folic acid intake and plasma folate concentration of 24 month-old rats.

(y = plasma folate, x = estimated folic acid intake)

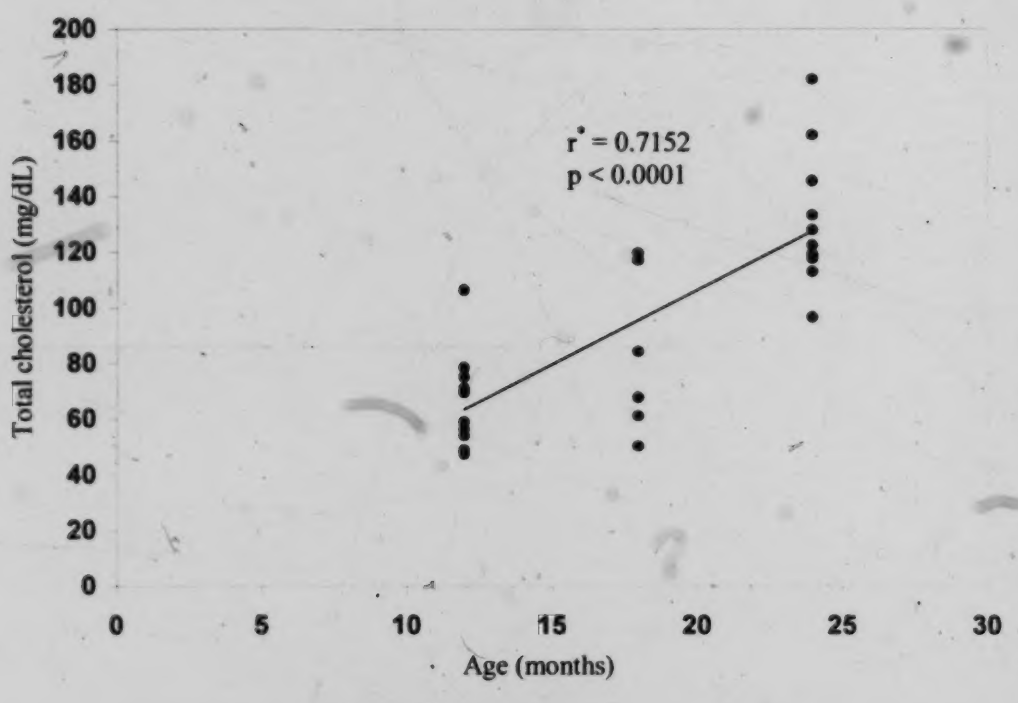


Figure 18: Correlation analysis of plasma total cholesterol concentration vs. age in 12, 18, and 24 month-old Fisher 344 rats not receiving supplemental dietary folic acid

(y = total plasma cholesterol concentration, x = age in months)

*Pearson correlation coefficient

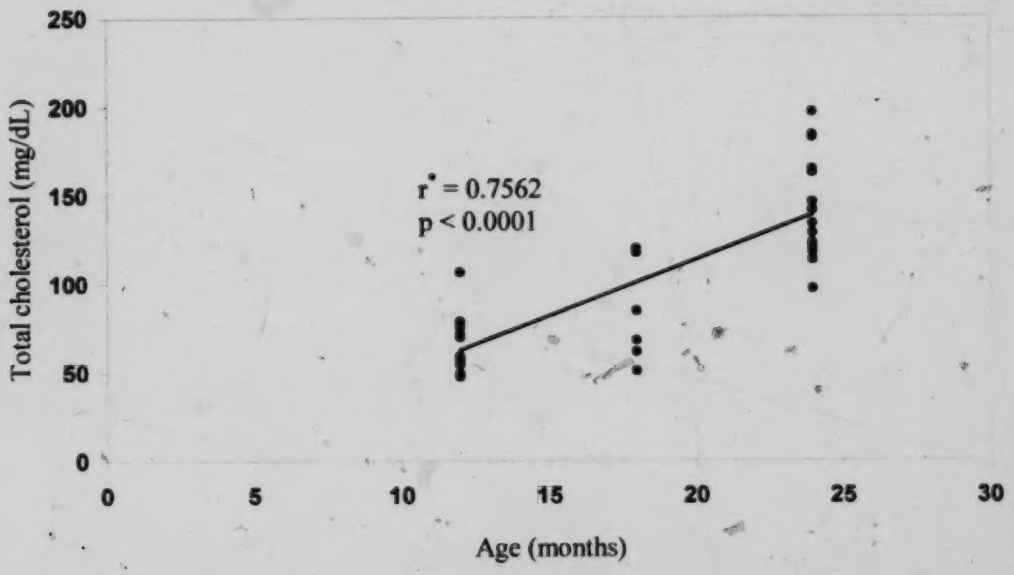


Figure 19: Correlation analysis of plasma total cholesterol concentration vs. age in 12, and 18 month–old Fisher 344 rats, as well as 24 month-old rats with and without supplemental intake of folic acid (35.7 mg/Kg) for four weeks

(y = total plasma cholesterol concentration, x = age in months)
*Pearson correlation coefficient

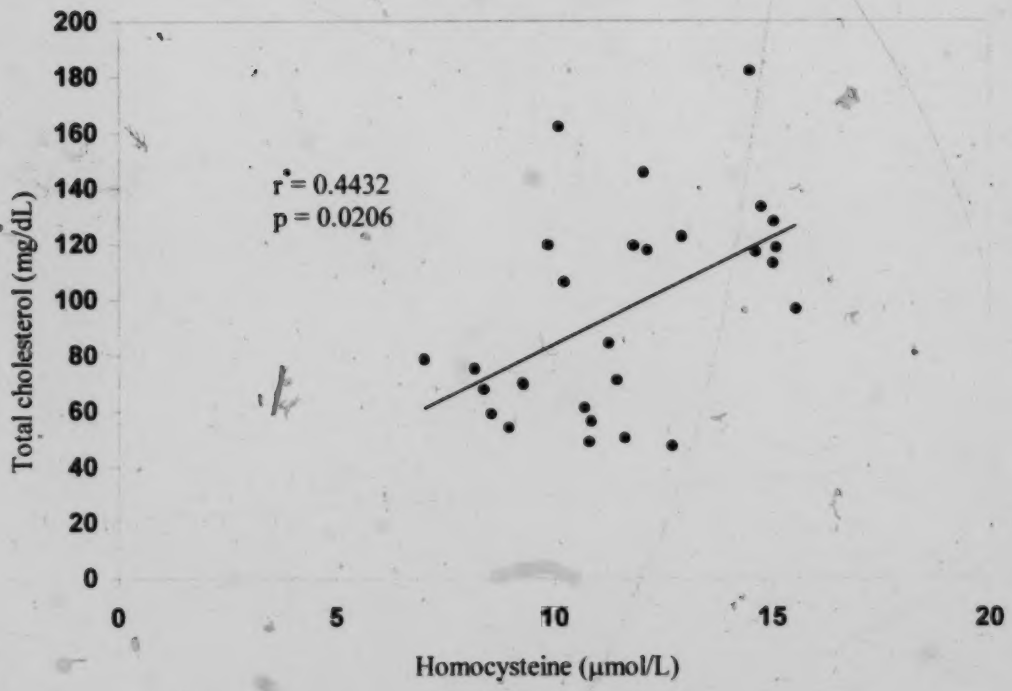


Figure 20: Correlation analysis of plasma total cholesterol vs. plasma homocysteine concentrations in 12, 18, and 24 month-old Fisher 344 rats not receiving supplemental dietary folic acid

(y = total plasma cholesterol concentration, x = plasma homocysteine concentration)
*Pearson correlation coefficient

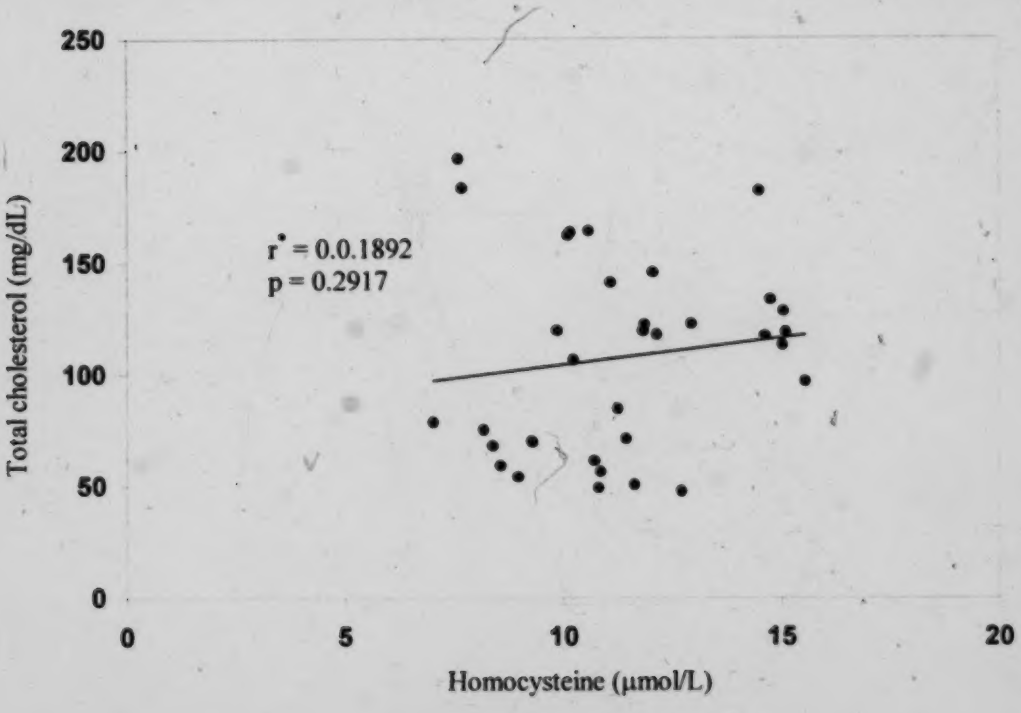


Figure 21: Correlation analysis of plasma total cholesterol vs. plasma homocysteine concentrations in 12, and 18 month-old Fisher 344 rats, as well as 24 month-old rats with and without supplemental intake of folic acid (35.7 mg/Kg) for four weeks

(y = total plasma cholesterol concentration, x = plasma homocysteine concentration)

*Pearson correlation coefficient

CHAPTER 5: DISCUSSION

The male Fisher 344 rat is a popular model for aging studies, accordingly, many of the age-associated changes of this strain have been documented (Barakat et al., 1989; Masoro, 1980; Masoro, 1992; Masoro et al., 1994). However, there have been few, if any, reports regarding any age-related changes in homocysteine metabolism in this, or any other, animal species. Hence, it is not known whether Fisher 344 rats experience an increase in plasma homocysteine with age, mimicking the increase found in humans. The present study is the first one to examine the relationship between homocysteine, cholesterol and the B-vitamins as response to aging in Fisher 344 rats.

The Fisher 344 rats were obtained from the National Institute of Aging (NIA), which breeds and maintains these colonies specifically for use in aging research. The maintenance of defined environmental conditions is an important step in limiting variability in the model and isolating the normal aging process. For example, determining the normal physiologic characteristics of aging requires that the animals remain free of infectious diseases; the latter can decrease the life span and increase the inconsistency in the model (Masoro, 1980). The Fisher 344 rats from the NIA colonies are maintained in a specially designed barrier facility to protect them from exposure to various pathogenic organisms throughout their life span. Additionally, all rats in the NIA colony receive the same type of food and water, thereby minimizing the variability that can result from differences in nutritional influences throughout the life span. Every effort was made to ensure that the age-related changes observed in the Fisher 344 rats in the current study were the result of normal physiologic processes.

The median length of life of barrier-maintained male fisher 344 rats given free access to food is approximately 28-29 months, thus, the rats used in the present study were between middle and the end of their natural life span (Masoro, 1980). The study design was not strictly longitudinal, however, differences between age groups can be considered to mirror age-related changes, as the rats were from the same colony, which was maintained under rigorously standardized conditions throughout their lifespan. Assessment of the general health status of the rats is essential for the differentiation of pathology-related changes and intrinsic aging effects (Bode and van, 1991). The hematological parameters suggest that the rats were generally healthy and free of infection. The age-related increases observed in MCH and MCHC are normal for Fisher 344 rats (Charles River Laboratories, 1984). No indicators of macrocytic or microcytic anemia were present,

indicating that there were no gross dietary deficiencies of iron, vitamin B_6, vitamin B_{12} or folate. Additionally, clinical chemistry profiles signify that the rats used in these experiments were apparently healthy.

In general, the 12 and 18 month-old rats gained weight throughout the course of the experiment, while the 24 month-old animals lost weight. However, there was no significant difference in food intake per unit body weight, thus all rats were receiving similar amounts of both methionine, the homocysteine precursor, and the B-vitamins involved in homocysteine metabolism (except where supplemental folic acid was provided). Generally, when given free access to food, these rats experienced a rapid increase in body mass during the first 8 months of life followed by a less rapid, linear increase until maximum body weight is reached at 20 months (Masoro, 1980). After 20 months there is normally a significant decrease in weight comprised of both lean and non-lean body mass (Masoro, 1980). Thus, the pattern of body weight change observed in the present study was normal and the weight loss was not indicative of ill health.

Determining the general health status of the rats is an important step when studying the effects of aging on rat plasma homocysteine levels, as homocysteine is known to increase in a number of disease states (Arnadottir et al., 1996; Gupta et al., 1998; Hernanz et al., 1999; Nedrebo et al., 1998; Refsum et al., 1989; Refsum et al., 1991; Roubenoff et al., 1997). In the present study, the homocysteine levels of apparently healthy Fisher 344 rats were found to increase in an age-related manner; plasma homocysteine concentrations were 28% higher at 24 months than at 12 months and there was a strong positive correlation between homocysteine and increasing age. This age-related increase in plasma homocysteine has not been previously reported to occur in rats; however, such a relationship has been described for humans. In both healthy subjects, and in those with cardiovascular disease there is a direct correlation between increasing age and plasma homocysteine concentrations (Andersson et al., 1992a; Brattstrom et al., 1994; Brattstrom et al., 1992; Jacques et al., 1999; Nygard et al., 1995; Robinson et al., 1995; Rossi et al., 1999; Selhub et al., 1993). Study subjects 80 years of age or older, in the Framingham cohort, had plasma homocysteine levels approximately 20% higher than subjects aged 67-74 years (Selhub et al., 1993) and Kark et al (1999) found a 25-30% increase in homocysteine concentrations in subjects aged 75 years old or older compared to subjects aged 50-64 years. Thus, the increase in homocysteine in aging rats appears to parallel the increase observed in humans during the latter portion of their life span. This age-related elevation in homocysteine levels is of particular

concern because it results in an increasing prevalence of hyperhomocysteinemia (>15 μmol/L) in the elderly.

Hyperhomocysteinemia is becoming increasingly recognized as a risk factor for cardiovascular disease, this association appears to be graded and remains independent of other established risk factors for vascular disease (Refsum and Ueland, 1998; Selhub, 1999). Cardiovascular disease is the leading cause of death and disability in developed nations and accounts for almost 40% of deaths in Canada (Statistics Canada, 1999). Although the development of atherosclerosis begins in childhood, there is a substantial elevation in the incidence of cardiovascular disease around 45 years of age for men and around 55 years of age for women (Heart and Stroke Foundation of Canada, 1997). Thereafter, the risk of developing cardiovascular disease continues to dramatically increase with age in both genders (Statistics Canada, 1999). This is of particular concern because the proportion of the Canadian population that is older than 65 is increasing rapidly and it is likely that the societal impact of cardiovascular disease will increase as the proportion of the population that is elderly expands. Therefore, the identification and treatment of modifiable cardiovascular disease risk factors, in this age group, may become increasingly important. Homocysteine is a prime candidate for this research because its increase in concentration with age and its persistence as a cardiovascular disease risk factor later in life indicate that it may be placing a substantial segment of the elderly population at increased risk for vascular disease. There are currently no reported studies that have longitudinally examined the age-related changes in homocysteine and the status vitamin B_6, vitamin B_{12} and folate concurrently or in the same individuals nor have there been any studies that have attempted to determine if this age-related increase in homocysteine can, or should be avoided.

The exact cause of the age-associated increase in plasma homocysteine levels has not been elucidated, but a variety of potential contributing factors have been identified. These potential factors include impaired activity of cystathionine-β-synthase, declining renal function, and poor intake, absorption and/or utilization of B-vitamins (Koehler et al., 1996; Nordstrom and Kjellstrom, 1998; Norlund et al., 1998; Selhub et al., 1993).

Rat plasma PLP typically decreases gradually from adulthood to old age (Cochary et al., 1990; Stabler et al., 1997). However, this pattern was not observed in the present study. This disparity may be due to the fact that the NIH-07 diet, used in the current investigation, provided approximately 50% more pyridoxine than the semi-synthetic diets used by others (Cochary et al.,

1990; Stabler et al., 1997). Thus, the long-term provision of higher levels of vitamin B_6 could have prevented the gradual decline in plasma PLP with increasing age.

There have been a few studies linking plasma vitamin B_6 levels to fasting homocysteine concentrations, however, most studies indicate that the influence of vitamin B_6 status is limited to the non-fasting state (Guttormsen et al., 1996). As fasting, rather than non-fasting or post-methionine load homocysteine levels were measured in the current study, the lack of correlation between plasma PLP and homocysteine concentrations is not unexpected. Even when adult rats are vitamin B_6 depleted, for a period of 6-9 weeks their fasting homocysteine levels do not become elevated (Miller et al., 1992). This relationship can be explained by the role of vitamin B_6 in homocysteine metabolism; when levels of methionine are high, homocysteine is directed towards its catabolic trans-sulfuration pathway for irreversible conversion to cysteine. There are two steps in this pathway; the first is the condensation of serine with homocysteine, via CBS to form cystathionine, and the second is the cleavage of cystathionine to cysteine and α-ketobutyrate via cystathionase (Stipanuk, 1986). Both of these enzymes require vitamin B_6, in the form of PLP, as a cofactor. The trans-sulfuration becomes impaired when vitamin B_6 status is sub-optimal. However, the disturbance is not necessarily manifested as hyperhomocysteinemia. If both vitamin B_{12} and folate are available in adequate amounts, homocysteine will be remethylated to methionine in the fasting state. It is only when high levels of methionine are present and exerting stress on the trans-sulfuration pathway that hyperhomocysteinemia will develop (Refsum et al., 1997). It has been reported that fasting homocysteine alone is unsuccessful in identifying >40% of subjects with impaired trans-sulfuration (Bostom et al., 1995). Thus, in order to thoroughly examine all of the vitamin-dependent pathways involved in homocysteine metabolism both fasting and post-methionine load homocysteine should be determined. However, the high plasma levels of PLP observed in the present study suggest that vitamin B_6 deficiency was not responsible for the age-related increase plasma homocysteine levels.

Another vitamin involved in homocysteine metabolism is vitamin B_{12}. Like PLP the plasma vitamin B_{12} concentrations of the rats in the present study did not decrease in an age-related fashion, rather they increased with age. This finding is in accordance with the observations of Koblin et al (1990) who measured vitamin B_{12} levels in Fisher 344 rats aged 2, 12 and 24 months and found no consistent linear trend in plasma vitamin B_{12} concentrations with respect to age. Instead they observed a slight, but non-significant, decrease in plasma vitamin B_{12} levels from 2 to 12 months of age followed by an increase (non-significant) from 12 to 24 months of age. They

also found that the vitamin B_{12} concentration in the liver increased significantly from 12 months to 24 months of age. One possible explanation for this age-related increase may be the high level of vitamin B_{12} in the NIH-07 diet. Based on the observation that 10 μg cyanocobalamin per Kg diet is insufficient to maintain adequate vitamin B_{12} status in rats, the American Institute of Nutrition Rodent Diet guidelines suggest that rodent diets should contain 25 μg vitamin B_{12} per Kg diet (Reeves et al., 1993). The diet used in the present study provided vitamin B_{12} at a level of 77.77 μg/kg diet; more than three times the recommended level. Unlike the other vitamins, vitamin B_{12} is partially fat-soluble and is stored in the liver. As a result of these properties the half-life of vitamin B_{12} is estimated to be greater than 400 days (Basu and Dickerson, 1996). Prolonged intake of the NIH-07 diet, with its high level of vitamin B_{12}, coupled with the long half-life of the vitamin in the body may result in the accumulation of this vitamin in the tissues. This may offer a likely explanation for the age-related increase in plasma vitamin B_{12} levels observed in the current study.

Although it does not appear to be important in old rats, vitamin B_{12} status is of particular importance in elderly humans. The prevalence of vitamin B_{12} deficiency in humans increases with increasing age. As well, there is a significant negative linear association between plasma concentrations of vitamin B_{12} and age (Lindenbaum et al., 1988; Tucker et al., 2000). Approximately 5-20% of the elderly population is estimated to be deficient in vitamin B_{12} due to a combination of poor dietary intake and impaired absorption resulting from atrophic gastritis or pernicious anemia (Stabler et al., 1997). The remethylation of homocysteine to methionine via methionine synthase requires vitamin B_{12} as a cofactor and methyltetrahydrofolate as a co-substrate. Both must be present in sufficient amounts if a buildup of homocysteine is to be prevented. The association between vitamin B_{12} status and homocysteine concentrations is not as strong as the association between homocysteine and folate, however, as the prevalence of vitamin B_{12} deficiency is so high in the elderly that it should not be overlooked as a possible contributing factor to age-related hyperhomocysteinemia (Selhub, 1999; Selhub et al., 1993). It is important that the divergent findings of rat and human aging studies, with regards to vitamin B_{12} status, be taken into consideration when attempting to extrapolate the results of rodent aging studies to humans.

The age-associated decline in plasma folate levels observed in the present study is in accordance with the reports of other rat studies (Horne et al., 1989b). Serum folate concentration of Wistar rats was found to be 50% lower in 30 month-old rats than in 2 month-old rats (Varela-Moreiras et

al., 1994). However, the change in folate status found by Varela-Moreiras et al. (1994) was not accompanied by a significant increase in homocysteine concentrations in the older rats as it was in the present study. Plasma concentrations of folate in the rats (Tables 10 and 14) were almost ten-fold greater than they are in humans, a finding that is consistent with other studies (Koblin et al., 1990; Stabler et al., 1997). The decrease in folic acid status with age is also compatible with reports of increased susceptibility of elderly humans to folate deficiency (Quinn and Basu, 1996). Subjects in the Framingham study cohort, ages 67-93 years, had a prevalence of inadequate folate status of approximately 30 % (Selhub et al., 1993). Both low B-vitamin concentrations and advancing age were associated with increasing plasma homocysteine concentrations in this cohort. Other investigators have estimated the prevalence of folate deficiency in the elderly to be anywhere from 2-20% (Quinn and Basu, 1996; Sauberlich et al., 1987). The reason for this decline in folate status with age is not fully understood. It was suggested that the ability to absorb folate decreases with age due to diminished capacity to hydrolyze polyglutamyl folates to the absorbable monoglutamyl form (Kesavan and Noronha, 1983). However it has since been demonstrated that old subjects absorb polyglutamyl folates as well as young subjects and intestinal conjugase activity does not change with age (Bailey et al., 1984). The ability of isolated hepatocytes to transport and concentrate folate from their incubation medium decreases with aging. However, as liver concentrations of folate do not change with age it is unlikely that this is a contributing factor in the age-related changes in plasma folate (Horne, 1989a). Another possible explanation for the decrease in folate status with age is a defect in the digestive phase that releases folate from food prior to the conjugase-dependent hydrolysis of polyglutamyl folates.

The age-related decrease in plasma folate, observed in the present study, reached a maximum by 18 months of age. However, homocysteine did not show a significant increase until 24 months of age. This would explain the lack of significant correlation between plasma homocysteine and folate concentrations (Table 11). It is possible that the functional vitamin deficiency, and thus the hyperhomocysteinemia, did not develop until folate concentrations were depressed for a prolonged period. Thus, it can be deduced that the homocysteine elevation associated with age may have resulted from a reduction in the amount of folate available for homocysteine remethylation to methionine. However, the potential influence of declining renal function or an unidentified age-related decrease in the activity of one the enzymes regulating homocysteine concentration cannot be ruled out (Ubbink, 1994).

Although most of the rats had serum creatinine in the normal range, serum creatinine explained the greatest proportion of homocysteine variance after age. This association between homocysteine and creatinine has also been observed by others (Herrmann et al., 1999; Koehler et al., 1996; Lalouschek et al., 1999). Renal uptake and metabolism of homocysteine has been demonstrated in a rat model but has not yet been observed in humans (Bostom et al., 1995). Thus, the association between creatinine and homocysteine has not been fully explained. An extra-renal link between homocysteine and creatinine has been hypothesized as a possible explanation for this association. More than half of the total methylations in which SAM is the methyl donor occur in the synthesis of creatine-creatinine for muscle, thus, as more creatinine is synthesized more homocysteine is formed from SAM (Koehler et al., 1996). However, this does not explain the striking elevations of homocysteine that routinely occur in patients with renal failure (Bostom and Lathrop, 1997a; Wollesen et al., 1999). Nor does it explain the impaired ability of patients with renal failure to eliminate homocysteine from plasma after peroral homocysteine loading (Guttormsen et al., 1997). It is possible that the association between homocysteine and creatinine found in the present study might reflect an age-related decline in renal function within the normal range of serum creatinine.

Renal clearance capacity substantially declines with age and in the present study serum creatinine increased with age, possibly indicating reduced glomerular filtration rate (Norlund et al., 1998). Many studies in elderly humans have investigated only compromised vitamin status as a main factor for hyperhomocysteinemia while failing to assess the impact of declining renal function as an important cause for hyperhomocysteinemia. It has been hypothesized that the common problem of declining renal function in the elderly is principally responsible for the increase in homocysteine concentrations associated with aging, however, studies designed to explore this hypothesis are limited (Brattstrom et al., 1994; Norlund et al., 1998). Both serum creatinine and plasma cystatin C, a marker for glomerular filtration rate, increase with age and are associated with increases in plasma homocysteine (Norlund et al., 1998). Plasma cystatin C has been found to have an even higher predictive value for homocysteine concentrations than other factors, such as age or serum creatinine, thus indicating that some of the increase in homocysteine observed with age could be partly explained by age-related decline in renal function (Norlund et al., 1998). It is important to recognize that, in the present study, homocysteine levels correlated more strongly with creatinine levels than with vitamin status, possibly indicating that the impairment in kidney function that occurs with age has more impact on plasma homocysteine than declining plasma folate levels.

Although plasma homocysteine concentrations did not correlate significantly with folate concentrations when animals from all age groups were considered, increasing the folic acid content of the NIH-07 diet by tenfold resulted in a reduction of homocysteine levels of 24 month-old rats by approximately 35% to a level that was similar to that found in the 12 month-old animals. Thus, the role of declining folate status as a contributor to the age-related increase in homocysteine cannot be ruled out merely because the two are not significantly correlated. It is likely that a combination of factors including both declining folate status and kidney function contributed to the age-associated increase in homocysteine. However, if diminishing renal function is partly responsible for the changes in homocysteine levels the present study gives some indication that, at least in the short term, this effect can be overridden by folate supplementation.

The ability of folic acid supplements to produce a decrease in plasma homocysteine levels in the general population has been clearly established. A recent meta-analysis of randomized trials concluded that daily supplementation of 0.5-5 mg folic acid would be expected to result in a 24% reduction in homocysteine levels, with an additional 7% reduction upon the inclusion of 0.5 mg supplemental vitamin B_{12} (Boushey et al., 1995). Human studies have also been carried out to determine whether or not vitamin supplementation can decrease plasma homocysteine levels in the elderly population. Intramuscular injection of a combination of 1.1mg vitamin B_{12}, 1mg folic acid and 5mg vitamin B_6 was found to reduce elevated homocysteine levels in a group of institutionalized elderly subjects (Naurath et al., 1995). The daily oral administration of 0.4-5.0 mg folic acid, for up to 90 days, to a group of elderly patients with coronary artery disease resulted in the normalization of elevated plasma homocysteine concentrations (Lobo et al., 1999). However, there is little known about the long term-effects of folic acid supplementation on homocysteine levels of the healthy elderly population, and the safety of such supplementation on a widespread basis is also questionable. Vitamin B_{12} deficiency is a common and often undiagnosed problem in the elderly population due to the high prevalence of atrophic gastritis and pernicious anemia (Stabler et al., 1997). The provision of folic acid supplementation to such individuals could promote purine synthesis and cell proliferation thus preventing the development of megaloblastic anemia, one of the first clinical manifestations of vitamin B_{12} deficiency (Bassler, 1997). If the vitamin B_{12} deficiency is allowed to progress it is feared that irreversible sub-acute combined degeneration of the spinal cord will be permitted to develop unnoticed (Bassler, 1997).

The strong age-related increase in plasma total cholesterol concentration, observed in the present study (Figures 16 and 17), is in accordance with the findings of others, as is the lack of significant age-related change in hepatic lipid concentrations and plasma triglyceride levels (Nakamura et al., 1989; Story et al., 1976). Similar age-dependent increases in plasma cholesterol have been reported in humans (Neves et al., 1997; Parini et al., 1999). The exact cause of this increase is not known but it has been hypothesized that alterations in hepatic lipid metabolism or intestinal lipid absorption with aging may be contributing factors (Nakamura et al., 1989; Uchida et al., 1996). There was a strong correlation between increasing cholesterol levels and increasing levels of plasma homocysteine and vitamin B_{12}. Vitamin B_{12} is regarded as a lipotropic factor as it is necessary for the secretion of lipids from the liver. Thus, the correlation between plasma cholesterol and vitamin B_{12} is not unusual (Zeisel et al., 1989). There is some evidence supporting an interaction between homocysteine and cholesterol, as homocysteine has the ability to stimulate the production and secretion of cholesterol in hepatic cell cultures (Lynn et al., 1998). Furthermore, methionine and cholesterol feeding act synergistically to raise plasma levels of homocysteine, cholesterol and triglycerides in rats and the combined effect of methionine and cholesterol feeding was much higher than the influence of feeding cholesterol or methionine alone, possibly indicating the importance of dietary factors, other than vitamins, on the outcome of hyperhomocysteinemia (Zulli et al., 1998a). Moreover, the combination of methionine and cholesterol feeding in rats has been found to act synergistically in promoting the disintegration of the elastic lamina when compared to the feeding of either agent alone (Zulli et al., 1998b). The results of the current study do not support a causative role for hyperhomocysteinemia in the age-related increase in total cholesterol levels. Folic acid supplementation ameliorated the age-associated increase in plasma homocysteine but did not alter cholesterol levels (Figures 18 and 19). Thus, the association between homocysteine and cholesterol disappeared with folic acid supplementation, but the association between age and cholesterol did not, therefore indicating the direct correlation between homocysteine and cholesterol was not independent of age. However, given the combined effect of homocysteine and cholesterol in promoting vascular damage, it is important to investigate the combined effects of cholesterol and homocysteine on cardiovascular disease as well as each agent alone (Zulli et al., 1998b).

This is one of the first studies to demonstrate the effect of folic acid supplementation on multiple facets of homocysteine metabolism in a properly controlled aging animal model. The confounding influences such as medication use, nutritional variation and social issues that often act as confounding factors in studies of aging humans were avoided. The absence of these confounding factors facilitated the elucidation of most accurate possible picture of the changes that occur, with increasing age, in plasma levels of lipids, homocysteine, homocysteine's metabolites, as well as the B-vitamins involved in its metabolism. Additionally, the present study contributed to the understanding of the true effects of folic acid supplementation on age-related hyperhomocysteinemia and hypercholesterolemia.

It was hypothesized that the plasma homocysteine concentration increases with age as a result of impaired folate status and that the age-related increase in homocysteine would be accompanied by an increase in lipid levels. It was further hypothesized that improving folate status would attenuate the age-related increase in homocysteine and cholesterol. As predicted, plasma concentrations of homocysteine did, indeed, increase from middle age to elderly Fisher 344 rats and this increase was similar to that observed in older humans. This provides the first evidence that the Fisher 344 rat, a common animal model for aging research, may be a valuable tool in studying age-related hyperhomocysteinemia. Despite *in vitro* evidence of an interaction between homocysteine and cholesterol production, the findings of the present study do not support this relationship. The association observed between the two appears to be strong, but upon further examination it seems that the association is largely the result of the age-dependent increase in both risk factors. Not surprisingly, both declining folic acid levels and increasing creatinine levels accompanied the elevation in homocysteine levels with age. These results indicate that both impaired kidney function and declining folate status may play a significant role in the development of age-related hyperhomocysteinemia. However, even in the face of high-normal creatinine levels, folic acid supplementation for a period of four weeks attenuated the age-associated increase in plasma homocysteine.

One notable limitation of the current study was the absence of a 12-month-old folic acid supplemented control group. The presence of such a group would have facilitated more in-depth statistical analysis and the exploration of any diet and age interactions. Furthermore, the NIH-07 diet provided to the rats in this study contained significantly more than the required amounts of dietary B-vitamins. As elderly humans often have borderline or even deficient vitamin intake, it would seem that a more representative study of the age-associated changes in homocysteine

metabolism should also include rats with marginal vitamin intakes. Additionally, the present study was limited to the supplementation of only one vitamin involved in homocysteine metabolism, thus it was not determined whether a supplemental regimen including vitamin B_{12} and/or vitamin B_6, in addition to folic acid, would be more effective than folic acid alone in ameliorating age-dependent hyperhomocysteinemia. However, even with these limitations, the study design did allow for the determination of the effects of folic acid supplementation on age-related hyperhomocysteinemia and hypercholesterolemia.

Despite the abundance of recent homocysteine research it still remains unknown whether hyperhomocysteinemia does, indeed, cause vascular disease and whether maintaining stable, low homocysteine levels will decrease disease risk. There are a number of long-term, prospective studies currently underway attempting to clarify this issue. However, as elderly people are at a higher risk than younger people for deficiency of folate, vitamin B_{12} and vitamin B_6, as well as hyperhomocysteinemia and vascular diseases, it may be advisable to consider some potentially preventive factors for this sector of the population. Elderly subjects with the lowest homocysteine levels are also either regular users of multivitamins and/or frequent consumers of fruit, vegetables and fortified breakfast cereals (Tucker et al., 1996). Thus, it may be beneficial to encourage older individuals to increase consumption of these products. This would present a safe, cost effective means of increasing B-vitamin intake and lowering homocysteine levels. Additionally, for elderly people with known cardiovascular disease risk factors or hyperhomocysteinemia resistant to changes in diet, a daily multivitamin containing at least 400 μg of folic acid and enough vitamin B_{12} to compensate for impaired absorption (500 mg) may be indicated (Moustapha, 1999).

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